Inducing mutations in bread wheat (*Triticum aestivum* L.) using chemical treatments.

By

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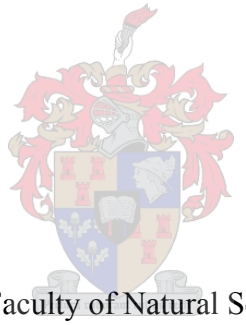
Masters of Science



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Declaration

By submitting this thesis/dissertation electronically, I **Kenneth Charles Mbwanji**, hereby declare that the entirety of the work contained therein is my own, original work, and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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Signature:

Abstract

Bread wheat (*Triticum aestivum* L.) production is hindered by a variety of abiotic stresses with drought being the most devastating. Mutation breeding through induced mutagenesis is one way for wheat breeders to adapt to the challenges posed by climate change. Especially, chemically induced mutagenesis showed promise in improving drought tolerance using different recommended mutagens at optimum concentrations and treatment durations. The aim of the study was to improve drought tolerance in wheat by means of chemical induced mutagenesis. In the study, the mutagenic properties of four chemicals, namely Sodium azide (SA, 0.1 mM, 1.0 mM and 10 mM), Ethyl methanesulfonate (EMS, 0.1%, 0.5%, 1.0% v/v), Maleic hydrazide (MH, 0.5 mM, 1.0 mM, 2.0 mM), and *N*-methyl-*N*-nitrosourea (MNU, 0.5 mM, 1.0 mM, 2.0 mM) at different concentrations and treatment durations (2h, 4h and 8h) were compared. To select for mutants that express drought tolerance, the M_1 plants were exposed to water stress. NMU and MH treated M_1 plants demonstrated zero survival rates, while a few of the plants treated with SA and EMS survived. In the study, treatments with 0.5% (EMS) 4h, 1% (EMS) 2h, 1 mM (SA) 2h and 1 mM (SA) 8h were considered optimum, since these treatments resulted in fertile plants. However, the chemically derived mutant wheat lines displayed a lower germination rate, delayed maturity, stunted growth and lower than average seed mass when compared to the control. The latter traits were also verified in the M_2 and M_3 generations. The M_2 and M_3 generations also displayed a shorter growth form and delayed maturity phenotype, but had higher germination rates and produced more seeds. Screening for drought tolerance conducted on the M_3 plants confirmed the tolerant phenotype found in the M_1 generation plants. Amplified fragment length polymorphism (AFLP) profiling was also conducted on the mutants using three primer combinations (MTT/ECG, MTG/ECT and MTG/EGC) in order to assess the extent and significance of the induced mutations. From the obtained data, it was revealed that SA 1 mM (2h) 16 had the highest number of induced total character differences (109) relative to the control of all the SA and EMS derived mutants, suggesting that a treatment with 1 mM SA for 2h induced more mutations than any other SA or any of the EMS treatments. Of the sequenced clones, only one revealed similarity to a *T. aestivum* isolate AAC/CTG7 scab resistance-linked

AFLP fragment gene sequence, an important disease of wheat, but due to time constrains this finding was not investigated further.

Opsomming

Die produksie van broodkoring (*Triticum aestivum* L.) word deur ‘n aantal abiotiese stressors beperk, met droogte die een met die grootste impak. Mutasie-teling deur middel van geïnduseerde mutagenese is ‘n manier waardeur koringtelers die uitdagings van klimaatsveranderinge kan oorbrug. Veral chemies-geïnduseerde mutagenese vertoon belofte vir die verbetering van droogte-toleransie deur middel van die gebruik van verskillende aanbevole mutagene teen optimale konsentrasies en blootstellings tye. Dus, die doel van die studie was om droogte-toleransie in koring deur middel van chemies-geïnduseerde mutagenese te bewerkstellig. In die studie is vier chemikalië teen verskillende konsentrasies dit is Natriumasied (SA, 0.1 mM, 1.0 mM and 10 mM), Etielmetaansulfonaat (EMS, 0.1%, 0.5%, 1.0% v/v), Malaathidrasied (MH, 0.5 mM, 1.0 mM, 2.0 mM), and *N*-metiel-*N*-nitrosourea (MNU, 0.5 mM, 1.0 mM, 2.0 mM) en blootstellings tye (2h, 4h en 8h) vergelyk. Om vir droogte-toleransie in die M_1 plante te selekteer, is hierdie plante aan waterstres-toestande blootgestel. NMU- en MH-behandelde M_1-plante het geen toleransie vertoon nie, aangesien alle plante gevrek het, terwyl SA- en EMS-behandelde plante oorleef het. In die studie het behandelings met 0.5% (EMS) 4h, 1% (EMS) 2h, 1 mM (SA) 2h en 1 mM (SA) 8h vrugbare plante gelewer en is as optimaal beskou. Die chemies-geïnduseerde mutante het egter ‘n laer kiemkragtigheid, vetraagde volwassewording, korter groeivorm en laer gemidddelde saadproduksie vertoon. Hierdie eienskappe is ook in die M_2- en M_3-generasies beoordeel. Die M_2- en M_3-generasies het ook die kort groeivorm en verlengde volwassewording vertoon, maar het meer kiemkragtigheid en ‘n groter saadproduksie vertoon. Droogte-toleransie wat by die M_3-plante geëvalueer is, het die toleransie wat in die M_1-generasie plante bevind is, bevestig. Geamplifiseerde fragment lengte polimorfisme (AFLP)-profiele is ook met drie inleier-kombinasies (MTT/ECG, MTG/ECT and MTG/EGC) op die mutante gedoen, en die mate en belangrikheid (indien enige) van die chemies-geïnduseerde mutasies, is gekwantifiseer. Vanuit die data kon afgelei word dat SA 1 mM (2h) 16 die meeste totale aantal karakter verskille (109) van alle SA en EMS-behandellinge, relatief tot die kontrole tot gevolg gehad het, wat aandui dat hierdie behandeling die meeste mutasies geïnduseer het. Van die klone, waarvan die basisvolgorde bepaal was, het slegs ee ooreenkoms aan ‘n *T. aestivum* isolaat AAC/CTG7 scab weerstand-gekoppelde AFLP-fragment geenvolgorde vertoon.

Scab is ‘n belangrike siekte van koring, maar as gevolg van ‘n tydbeperking kon hierdie bevinding nie verder ondersoek word nie.

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List of abbreviations

%	Percentage
µl	Microliter
µM	Micromolar
AFLP	Amplified Fragment Length Polymorphism
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
CIMMYT	Centro Internacional de Mejoramiento de Maiz y Trigo (International Maize and Wheat Improvement Center)
cm	Centimetres
CO_2	Carbon dioxide
dH_2O	Distilled water
DN	*Diuraphis noxia*
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
E.coli	*Escherichia coli*
EMS	Ethylmethanosulfonate
FAO	Food and Agriculture Organisation
FHB	Fusarium head blight
g	Grams
GD	Genetic distance
hr	Hour(s)
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	Kilobase
KCL	Potassium chloride
LB	Lubia Broth
M	Molar
M_0	Mutagenic parent (starting material)
M_1	Mutagenic lines first generation

M_2	Mutagenic lines second generation
M_3	Mutagenic lines third generation
$MgCl_2$	Magnesium chloride
MH	Maleic hydrazide
min	Minutes(s)
ml	Milliliter
mm	Millimetre
mM	Millimolar
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
NMU	*N*-Nitroso-*N*-methylurea
ºC	Degrees celsius
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
SA	Sodium azide
sec	Second(s)
SOC	Super Optimal Broth
SU	Stellenbosch University
TF	Transcriptional factor
T_m	Melting temperature
U	Unit(s)
V	Volts
v/v	Volume/Volume
w/v	Weight/Volume

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Chapter 1: Introduction

Bread wheat (*Triticum aestivum* L.) is an economically important crop with a global output of 681 million tonnes in 2011 (Brenchley *et al*. 2012). According to the Food and Agriculture Organization (FAO), the estimated global wheat production in 2013 stood at 695 million tonnes, 5.4% up from 2012 and about 6 million tonnes less than 2011 (FAO 2013). Based on current consumption, wheat production is expected to reach 860 million tons per annum by 2030 (Tolmay 2006), making it an important food crop and thus has been of interest to plant breeders (Rana *et al*. 2013). Due to an increase in world population, wheat grain production must increase over the next decade to meet the demands of a rapid growing human population (Gasperini *et al*. 2012, Edgerton 2009). As a result of these demands, wheat has been broadly studied in order to improve a wider range of desired agronomic traits located across its genome (Lagudah *et al*. 2001). As the world population continues to grow and water resources for crop production decline, the development of drought-tolerant cultivars and water-use-efficient crops is a global concern (Barnabas *et al*. 2008).

Like most other cereal grains, improving wheat performance in moisture- and temperature-stressed environments is amongst the most serious challenges hampering global wheat production (Zhou *et al*. 2012, Hu *et al*. 2013). Normally a crop will show its maximum yield potential in a stress-free environment, while this potential will greatly be reduced under environmental stresses (Rana *et al*. 2013). This is especially true in Sub-Saharan Africa, where existing levels of drought and heat stress are expected to worsen in many areas due to climate change and rise in temperatures, and where smallholder farmers have limited options for adapting to increased levels of environmental stresses. Unlike other abiotic stresses, drought causes most adversities and threats as it directly reduces the growth and development of crop plants by disturbing and interfering with the normal biochemical processes and gene expression (Rana *et al*. 2013, Reynolds *et al*. 2010, Hu *et al*. 2013).

The key approach in mutation-based breeding is to improve the well-adapted crop varieties by changing major trait(s), which determine their performance or productivity. In several mutation-derived varieties, the altered traits have resulted in synergistic effects on increasing the quality and productivity of the crop such as nutrient, tolerance to stress factors and yield related traits

(Ahloowalia *et al*. 2004). Many mutation-derived plants have made an impact and will continue to have an increasing role in creating crop varieties with improved traits such as drought tolerance. Drought tolerance and plant improved performance under water stress conditions have been demonstrated in previous studies through the application of different mutagens (Ahloowalia *et al*. 2004). The aim of the present study was to improve drought tolerance in wheat by means of chemical induced mutagenesis. The objectives were firstly; using different chemical mutagens, develop and select through drought screening procedures the derived mutant wheat lines associated with drought tolerance. Secondly; to profile these mutagenic wheat lines using AFLP in order to assess the extent of induced mutations.

Chapter 2 of this thesis is comprised of an overview of literature pertaining to wheat response to abiotic stresses, with emphasis on the adaptive strategies of the wheat plant to environmental stresses. An insight to the functional analysis of drought responsive and drought tolerant genes is also reviewed. The inception and the implication of mutation breeding with its application in improving crop production is briefly described in this chapter.

Chapter 3 describes the development of mutagenic wheat lines using four mutagens namely; Sodium Azide (SA) , Ethylmethanosulfonate (EMS) , *N*-Nitroso-*N*-methylurea (NMU), and Maleic Hydrazide (MH), at different concentrations and treatment durations. Phenotypic screening was perfomed to select putative mutants related to drought tolerance.

Chapter 4 analyses the derived mutant wheat lines using AFLP in order to assess the extent of induced mutations that had been introduced by means of chemical induced mutagenesis.

Chapter 5 consists of a summary describing the significance and main findings of this study.

Appendix contains relevant information used during calculation of genetics distances.

1.1. Preface

The findings presented in this thesis represent the results of a study undertaken between February 2012 and July 2014 in the Department of Genetics, University of Stellenbosch, under the supervision of Prof. AM Botha-Oberholster

The following outputs resulted from the thesis:

Conference poster presented:

Mbwanji, KC., and Botha. A-M (2012) Improvement of drought tolerance in wheat. Proceedings of the South African Genetics and South African Society for Bioinformatics and Computational Biology SAGS / SASBCB 2012. PP50.

Seminar talk:

Improvement of drought and heat tolerance in wheat (*Triticum aestivum* L) using chemical induced mutagenesis. Proceedings of the food security initiative, Stellenbosch University 2012.

Workshop oral presentation:

Improvement of drought and heat tolerance in wheat (*Triticum aestivum* L) using chemical induced mutagenesis. Proceedings of the National Research Foundation (NRF) bilateral (RSA/KENYA) bioinformatics workshop, Stellenbosch University 2014.

1.2. Reference list

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Chapter 2: Literature review

2.1. Bread wheat (*Triticum aestivum* L.)

The grass family (Poaceae), including wheat (*Triticum aestivum* L.), is of particular interest and essential to human survival. Global human populations rely on grasses especially bread wheat for a major portion of their energy intake (Kellog 2001), and as a primary source of vitamins and minerals. In 2011, bread wheat accounted for 20% of the calories consumed by humans worldwide (Brenchley *et al*. 2012). Through milling, wheat flour is produced which is then used in baking breads, making cakes, biscuits, breakfast cereal, preparing pasta, noodles and couscous food (Cauvain 2003). Bread wheat is also used in fermentation to make beer and other alcoholic beverages as well as for biofuel production (Doward 2010). Livestock which contribute a great portion of proteins for human consumption is also raised on diets partially or entirely of wheat (Kellog 2001). Consequently, both livestock and human wheat consumption plays a substantial role in supporting global calorie intake (Tang *et al*. 2012).

Wheat is one of the first cereals known to have been domesticated by man (Lev-Yadun *et al*. 2000) and archaeological records suggest that the first wheat domestication took place in the region of the Fertile Crescent (Modern Day Middle East) and the Nile Delta, 8000 BCE (Lev-Yadun *et al*. 2000). Recent findings narrow the first domestication of wheat down to a small region of south-eastern Turkey (Lev-Yadun *et al*. 2000). The spread of agriculture and settled societies is significantly associated with bread wheat cultivation and its domestication (Brenchley *et al*. 2012). Today, bread wheat is one of the most widely cultivated and important cereal crops (Garg *et al*. 2012, Shewry 2009), cultivated in nearly all parts of the world, with cultivation areas ranging from 45^{o}S in Argentina and 67^{o}N in the Scandinavian and Russian regions, as well as to the elevated subtropical and tropical regions of Africa and Asia respectively (Garg *et al*. 2012, Shewry 2009).

The ability of wheat to self-pollinate hugely facilitated the diversification of domesticated wheat varieties. Additionally, polyploidy is another factor that contributed directly to wheat`s evolution (Leitch and Bennett 1997). Allopolyploids are the result of hybridization of diploid nuclear genomes from two or more different ancestral species (Leitch and Bennett 1997). As a result of polyploidy, *Triticum* species have been successfully established throughout the world in diverse

environments (Hancock 2004, Lev-Yadun *et al*. 2000). Polyploidy provides even a greater advantage considering that most important crops such as wheat, cotton, coffee, potato, sugarcane and maize are polyploids (Leitch and Bennett 1997). Three ploidy levels namely: diploid, tetraploid and hexaploid wheat species have been domesticated at different periodical stages (Hancock 2004). Diploid wheat species are represented by einkorn (*Triticum monoccum*) which possess two complements of seven chromosomes (AA) 2n =14 (Hancock 2004). Most tetraploid wheat are represented by a number of sub-species, with emmer and durum wheat being the most common species. Driven by natural selection, the establishment of diploids and tetraploids took place in the wild long before domestication of bread wheat (Hancock 2004).

Bread wheat (*Triticum aestivum* L.) is an allopolyploid (allohexaploid; 2n = 6x = 42) with genome constitution; AABBDD, comprised of three distinct but yet closely related sub-genomes i.e. A, B and D as described in figure 2.1. These genomes are derived from three different progenitor species through the hybridization and somatic doubling of *T. urartu* (AA) with a B genome diploid of unknown origin forming a cultivated tetraploid emmer or durum (AABB), and consequent hybridization with a D genome wild diploid goat grass such as *Aegilops tauschii* (DD) (Gill *et al*. 1991, Devos and Gale 1997, Erayman *et al*. 2004, Kubaláková *et al*. 2002, Lagudah *et al*. 2001, Brenchley *et al*. 2012**).** The three diploid progenitor genomes radiated from a common Triticeae ancestor between 2.5 and 4.5 million years ago, while AABB tetraploids arose less than 0.5 million years ago. Nucleotide diversity in the DD and AABB genomes is significantly reduced compared with the ancestral populations, signifying a major diversity bottleneck on the transition to cultivated lines (Brenchley *et al*. 2012).

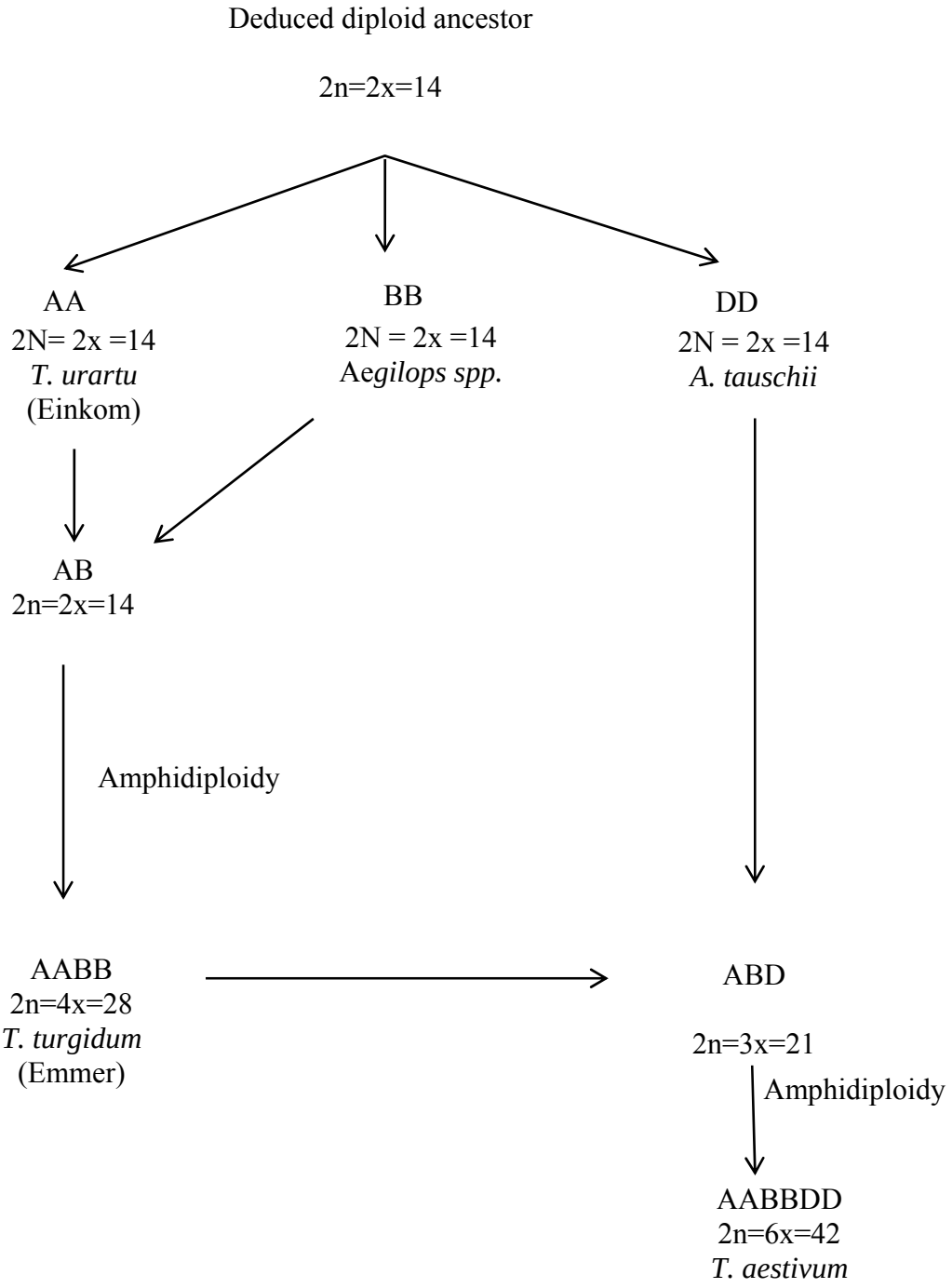

Figure 2.1: Shows the origin of wheat (*Triticum aestivum* L.) genome`s constitution AABBDD, derived from three different progenitor species (Gill et al. 1991, Devos et al. 1997, Erayman et al. 2004, Kubaláková et al. 2002, lagudah et al. 2001).

2.2. Abiotic stresses in crop production

Environmental stress factors such as drought, elevated temperatures and rising CO_2 influence plant development and pose an increasing threat to sustainable agriculture (Ahuja *et al*. 2010). Drought and temperature are the two most important abiotic stress factors, as a result of climate change (Barnabas *et al*. 2008, Reynolds and Trethowan 2007). Under humid environments, high temperatures are detrimental to bread wheat and such temperature elevations also attract temperate pests and diseases especially under humid environments, thus imposing an even greater threat to crop production (Reynolds and Trethowan 2007).

Multiple environmental stresses commonly occur simultaneously in nature. Drought and moisture deficit in conjunction with heat and/or high temperature are examples of typical multiple stress combinations (Ahuja *et al*. 2010, Barnabas *et al*. 2008). The impacts of multiple environmental stress combinations are different, but in all cases negative and always results in a decline in yield quantity and crop quality. This occurs especially during the process of grain filling and the accumulation of reserve nutrients in the developing and maturing grain (Barnabas *et al*. 2008).

Even though multiple abiotic stresses are common in nature, few studies investigating the plant responses to multiple environmental stresses have been conducted (Barnabas *et al*. 2008). A focus on molecular, physiological and metabolic aspects of a combination of stresses are therefore required to facilitate the development of crops with enhanced tolerance to stress conditions (Mittler 2006, Ahuja *et al*. 2010, Barnabas *et al*. 2008).

The concept of climate change, its related consequences and how plants cope with constantly changing climate, is still knowledge to be harnessed and fully understood, as most future predictions are based on the present climate patterns (Challinor *et al*. 2007, Ahuja *et al*. 2010). Regardless of the situation, improving crop performances grown under different forms of environmental stresses is still amongst the most serious challenges facing global agriculture today.

2.3. Adaptive strategies of plants to abiotic stresses

In the course of evolution, plants, as sessile organisms, have developed appropriate adaptive strategies to ensure their reproduction and survival even under suboptimal conditions (Barnabas *et al*. 2008). To cope with climate change calamities, plants have evolved different mechanisms to sense changes rapidly and adapt accordingly. Understanding these mechanisms under continuously changing environmental conditions has been a subject of great interest for many decades (Ahuja *et al*. 2010). Examples of the complicated response mechanisms include; the phytohormones to respond and perceive adverse external signals that have been developed by plants (Han *et al*. 2012). Moreover, the plant`s adaptive strategies to stress are ultimately coordinated and perfected by adjusting growth, development, molecular and the plant`s cellular activities (Zhang *et al*. 2005).

Plants use specific strategies to cope with drought stress and these are subdivided into dehydration avoidance, tolerance and escape. Dehydration avoidance, occurs when there is maintenance of a favorable plant water status during stress, maybe as the result of maximized water uptake e.g. by increased root growth or minimized water loss through stomatal closure, reduced leaf area, senescence of older leaves, etc. While tolerance to low water potential manifests as the maintenance of a plant`s function at limited water supply which may involve osmotic adjustments, but may also be the result of rigid cell walls or small cells (Barnabas *et al*. 2008). The heat tolerance of plants is a complex trait, most probably controlled by a multitude of genes, such as the terminal components of the signal transduction pathway represented by heat stress transcription factors (HSFs) that are responsible for mediating the activation of genes responsible for drought stress (Barnabas *et al*. 2008). On the other hand, escape strategies mainly rely on successful reproduction before the inception of severe stress, by means of a shortened life cycle, a higher rate of growth and/or the efficient storage and use of reserves for seed production (Barnabas *et al*. 2008).

A plant`s growth form (architecture) is another major agronomic benefiting factor as it controls the adaptability of a plant to cultivation, grain yield etc. (Gasperini *et al*. 2012). A key component of plant architecture is the height of the plant which is mainly determined by stem elongation. In wheat, the internode elongation is regulated by genes involved in gibberellin (GA) and brassinosteroid (BR) biosynthetic or signaling pathways (Gasperini *et al*. 2012). Due to severe abiotic stress, plants would react by delaying plant growth, plant development and subsequently delay flowering. Since dwarfism can resort as a "cost-saving" mechanism, it is thus an important trait for wheat breeders that targets drought tolerance in their varieties (Rana *et al*. 2013).

The physiological responses to drought trigger extensive changes in gene expression incurring adjustments in the proteomic and biochemical machinery (Ahuja *et al*. 2010). The plant`s responses to drought stress depend on genotype, the length and severity of water loss, the age and stage of development, as well as the organ and cell type (Barnabas *et al*. 2008).

Tolerance improvement of wheat cultivars to environmental stress could increase grain yields and provide a secure food source to those living on land that is marginally cultivable. However, due to its hexaploid genomic structure and large genome, genetic studies on wheat are challenging (Brenchley *et al*. 2012), especially those related to mechanisms involved in tolerance to environmental stresses. However, the cloning and functional validation of stress tolerance genes continues to provide a deeper understanding of the networks of signaling in response to stress in wheat (Du *et al*. 2013), which may eventually lead to the development of more stress-tolerant crops. Water limiting conditions greatly affect wheat productivity and therefore breeding for drought tolerance in wheat is an important area of research that is actively being pursued by various research teams across the world. A thorough understanding of the molecular mechanisms underlying the response to abiotic stress responses is however necessary to mediate a successful outcome during the genetic improvement of stress tolerance in wheat (Zhou *et al*. 2012, Hu *et al*. 2013).

2.3.1. Genes that confer drought stress tolerance in bread wheat

The advent of genomics has offered a comprehensive profiling of the changes in gene expression as a result of their exposure to drought and thus far many genes have been identified to be involved in drought response mechanisms in wheat (Table 2.3.1). However, as most of these genes have only been studied in model plants mostly being *Arabidopsis* and tobacco, a lot is still to be learned about the functions of these genes in bread wheat (Rana *et al*. 2013).

Stress-responsive genes can generally be classified into two groups, the first group comprises of functional proteins and enzymes that take part in osmotic stress protection and tolerance e.g. membrane transporters, water channels, antioxidative enzymes, osmotin, etc. (Garg *et al*. 2012). The second group includes; mainly the protein kinases and transcription regulators that collectively regulate the expression of numerous downstream genes and signal transduction (Garg *et al*. 2012). The transcription activators are considered the most popular and common targets for crop improvement as they interact and bind specifically to *cis* elements found in the promoter regions of several stress responsive genes (Morran *et al*. 2011, Garg et. al 2012). This in turn switches on their expression granting stress tolerance to crop plants (Morran *et al*. 2011, Garg et. al 2012).

Based on sequence analysis in model species, over 50 families of different transcription factors (TFs) have been identified in plants (Tang *et al*. 2012) such as: Dehydration-responsive element-binding proteins (DREB), Myeloblastosis (MYB) etc. The majority of these have been shown to be involved in the regulation of drought response in plants (Morran *et al*. 2011). Furthermore, several reports indicate that TFs are necessary and essential for plant development and vital in the conversion of stress signal perception to stress-responsive gene expression (Tang *et al*. 2012).

Table 2.3.1: A brief summary of functionally characterized genes involved in conferring drought tolerance and associated stresses in wheat.

Gene Family	Gene symbol	Abiotic stressors	References
Dehydration-responsive element-binding proteins (*DREB*)	*DREB1, DREB2,* CBF	Drought, low temperature, high salt, and extreme heat	Liu *et al*. 1998; Rana *et al*. 2013; Morran *et al*. 2011; Huseynova and Rustamova 2010; Sakuma *et al*. 2006
Late embryogenesis abundant (*LEA)*	*LEA, cor14b*	Drought, cold acclimation, salt stress, and extreme heat	Morran *et al*. 2011; Battaglia *et al*. 2008; Lopato and Langridge 2011; Rana *et al*. 2013
WRKY-type transcription factors (*WRKY)*	*TaWRKY2, TaWRKY19*	Drought and salt stress	Niu *et al*. 2012; Rana *et al*. 2013
Reduced height (*Rht)*	*Rht, Rht-B1b, Rht-D1b, Rht8*	Drought	Rana *et al*. 2013; Gasperini *et al*. 2012
Plant-specific NAC (NAM/ATAF/CUC) transcription factors (TFs)	*TaNAC2a, TaNAC4a, TaNAC6, TaNAC7, TaNAC13, TaNTL5*	Dehydration, salinity and low temperature	Tang *et al*. 2012; Rana *et al*. 2013
Myeloblastosis oncogenes	*MYB, TaMYB30 , TaMYB30-B, TaMYB2, TaMYB33*	Dehydration and salt stress	Zhang *et al*. 2010; Rana *et al*. 2013; Garg *et al*. 2012
Wheat expansis protein	*EXPB, TaEXPB23*	Water stress	Rana *et al*. 2013; Han *et al*. 2012
Aquaporin proteins	*AQP, TaAQP7*	Water stress	Rana *et al*. 2013; Zhou *et al*. 2012
Sucrose non-fermenting1-related protein kinases 2	*SnRK2, TaSnRK2.8*	Drought, salt stress and cold stress	Zhang *et al*. 2010; Rana *et al*. 2013
Abscisic acid (ABA)-stress and ripening-induced proteins	*ASR, TaASR1*	Drought	Hu *et al*. 2013

DREB

The first gene family that confers tolerance to drought is the dehydration-responsive element-binding proteins (*DREB*). DREBs are induced by abiotic stresses such as drought, low temperature (Liu *et al*. 1998, Rana *et al*. 2013, Morran *et al*. 2011), high salt and extreme heat (Morran *et al*. 2011) resulting in transcriptional up-regulation (Liu *et al*. 1998, Rana *et al*. 2013, Morran *et al*. 2011). Using *Arabidopsis*, six DREB transcription factors which include two *DREB2* and four *DREB1/CBF* genes have been identified (Morran *et al*. 2011). All *DREB* genes feature three conserved regions, a EREBP/AP2 DNA binding domain, an N-terminal nuclear localization signal and conserved Ser/Thr rich region adjacent to the EREBP/AP2 domain. DREB TFs play key roles in the plant stress signaling transduction pathway and activate the expression of many stress inducible genes (Huseynova and Rustamova, 2010, Lopato and Langridge 2011). In wheat, *Dreb1* genes are located on chromosomes 3A, 3B and 3D (Rana *et al*. 2013, Huseynova and Rustamova, 2010).

LEA

Late embryogenesis abundant (*LEA*) genes are active during the maturation of embryos and desiccation of seeds in both the embryo and endosperm. They are also induced by drought, cold and salt stresses in vegetative tissues (Morran *et al*. 2011). The elevated levels of particular wheat DREB factors have shown to activate and regulate the expression of specific *LEA* genes such as a nuclear *cor14b* responsible for drought stress in wheat (Battaglia *et al*. 2008, Morran *et al*. 2011, Lopato and Langridge 2011, Rana *et al*. 2013). In wild-type plants, *LEA* genes are active in tissues containing high levels of abscisic acid (ABA), such as grain, and are normally induced by a range of abiotic stresses. Expression of *LEA* genes is higher in stress tolerant varieties than in stress sensitive varieties. During cold acclimation, some *LEA* genes express to very high levels. In wheat, products of these genes are often quite hydrophobic and are involved in the direct protection of cell components, such as proteins, membranes, and organelles, from damage caused by dehydration (Lopato and Langridge 2011, Morran *et al*. 2011, Rana *et al*. 2013).

WRKY

WRKY-type transcription factors are involved in several developmental and growth stages of plant response and they have been reported to take part in the regulation of abiotic stress tolerance in transgenic *Arabidopsis*. Transgenic *Arabidopsis* containing and overexpressing *WRKY* genes, *TaWRKY2* and *TaWRKY19* showed drought and salt stress tolerance (Niu *et al*. 2012, Rana *et al*. 2013) when compared with controls.

Rht

Reduced height (*Rht*) genes are solely responsible for short stature in wheat which the plants may sometimes need for their survival. Previous studies revealed a close positive correlation between these genes and drought tolerance (Rana *et al*. 2013). Recently, the dwarfing wheat genes *Rht-B1b, Rht-D1b* and *Rht8* have been identified (Gasperini *et al*. 2012, Rana *et al*. 2013). Morphological analyses show that the semi-dwarf phenotype of *Rht8* lines is due to shorter intermodal segments along the wheat culm, achieved through reduced cell elongation. *Rht8* is one of the few genes to shorten wheat culms and improve lodging resistance without penalizing grain yield. It is described as a weak allele of a gene for height promotion on the short arm of chromosome 2D (Gasperini *et al*. 2012).

NAC

The Plant specific NAC (NAM/ATAF/CUC) transcription factors have been reported to play a role in diverse stress responses and developmental processes. The rice stress-responsive *NAC* gene, when incorporated in wheat, conferred tolerance to drought and salt stress (Rana *et al*. 2013). Molecular characterization of novel *TaNAC* genes namely *TaNAC2a, TaNAC4a, TaNAC6, TaNAC7, TaNAC13* and *TaNTL5* in wheat and overexpression of these genes in tobacco also conferred drought tolerance (Tang *et al*. 2012). These new genes encoding NAC TFs in *Triticum aestivum* are classified into three groups: stress-related NACs, development-related NACs and NTLs (membrane-associated TFs belonging to NAC) by phylogenetic analysis. A novel NAC TF gene *TaNAC8* and *TaNAC4* in wheat, responds to stripe rust pathogen infection. On the other hand, *TaNAC69* from the NAC superfamily of TFs is upregulated by abiotic stresses in wheat and recognizes two consensus DNA-binding sequences. Likewise,

transgenic bread wheat overexpression of *TaNAC69* enhances transcript levels of stress upregulated genes and dehydration tolerance (Tang *et al*. 2012)

MYB

Myeloblastosis oncogenes (*MYB*) take part in several processes of growth and developmental stages of plants as well as in response to stress. In wheat, *TaMYB30* and *TaMYB30-B* genes have been identified whereby both encode for R2R3-type MYB proteins and confer drought tolerance in transgenic *Arabidopsis* (Rana *et al*. 2013). Other wheat genes that have been reported to confer tolerance in wheat against drought stress include the *TaMYB2* (Garg *et al*. 2012, Rana *et al*. 2013) and *TaMYB33* genes. The later has been reported to be involved in the detoxification of reactive oxygen species (ROS) (Rana *et al*. 2013). Among abiotic stresses, MYB2 homologs from *Arabidopsis* were found to be induced in response to dehydration and salt stress (Garg *et al*. 2012), suggesting their important role in stress responsive gene regulation.

EXPB

Wheat expansins protein (*EXPB*) is regarded as a cell wall protein that is generally accepted to be a main regulator of cell wall extension during plant growth (Rana *et al*. 2013). They act by mediating pH dependent wall loosening, by disrupting hydrogen bonds between cellulose and matrix glycans. The regulation of cell extension is critical for plant growth and stress resistance. The plant cell wall determines the cell shape, and is the main barrier against abiotic and biotic stresses. Expansins are encoded by a large gene superfamily, including expansins (*EXP A*), b-expansins (*EXP B*), expansin-like A (*EXL A*), and expansin-like B (Han *et al*. 2012). In wheat, the expression of *TaEXPB23* corresponds to water stress and coleoptile (Han *et al*. 2012). Transgenic tobacco overexpressing *TaEXPB23* showed enhanced water retention ability (WRA) and decreasing osmotic potential. The *TaEXPB23* gene may be used in wheat cultivars to improve water retention ability (Rana *et al*. 2013).

AQP

When a plant is under stress the symplastic pathway using aquaporin (AQP) proteins is regarded more efficient in regulating water transport across the membranes. AQP are a large family within the major intrinsic protein (MIP) superfamily and are known be involved in the transport of water and other small molecules through biological membranes. Many *AQP* genes have been identified from different plant species including 35 from *Arabidopsis*, 36 from maize and 33 from rice (Rana *et al*. 2013, Zhou *et al*. 2012). Aquaporin (AQP) proteins are vital for plants to contest stress caused by water scarcity. Overexpression of *TaAQP7* confers drought stress tolerance in wheat cultivars which showed enhanced expression of the gene (Rana *et al*. 2013).

SnRK

Sucrose non-fermenting1-related protein kinases 2 (SnRK2) plays a vital role in abiotic stress signaling in plants. *TaSnRK2.8* has been reported as a regulatory factor, involved in many stress response pathways. Overexpression of *TaSnRK2.8* in transgenic *Arabidopsis* showed enhanced tolerance to drought, salt and cold stresses (Zhang *et al*. 2010). Based on these observations, this gene can therefore be utilized in wheat improvement against drought stress (Rana *et al*. 2013). In a study conducted by Zhang *et al*. (2010), *TaSnRK2.8*, a SnRK2 member in wheat, was cloned and its function under multi-stress conditions characterized. Subcellular localization showed the presence of *TaSnRK2.8* in the cell membrane, cytoplasm and nucleus. Expression pattern analyses in wheat revealed that *TaSnRK2.8* was involved in response to PEG, NaCl and cold stresses, and possibly participates in Abscisic acid (ABA) dependent signal transduction pathways (Zhang *et al*. 2010).

ASR

Abscisic acid (ABA)-stress and ripening-induced (ASR) proteins have been suggested to be involved in abiotic stresses. Overexpression of *TaASR1* in tobacco resulted in increased drought tolerance. Transgenic tobacco containing this gene showed ROS scavenging and higher level of relative water content, superoxide dismutase and catalase (Rana *et al*. 2013). The first wheat *ASR* gene, *TaASR1* is an ortholog of *OsASR5* (Os11g06720) that has been cloned and characterized. *TaASR1* transcripts increased after treatments with PEG6000, ABA and H_2O_2. Studies have

shown that *TaASR1* functions as a positive factor under drought/osmotic stress, involved in the regulation of ROS homeostasis by activating the antioxidant system and transcription of stress-associated genes (Hu *et al*. 2013).

2.4. Chemical induced mutagenesis in crop production

A significant proportion of environmental pollutants irrespective of their abiotic or biotic nature can exert genotoxic stress to plants that are incapable of physically escaping the potentially harmful environment. Besides ionizing and ultra-violet (UV) radiations, several abiotic stresses such as extreme temperature, water, salt, metal, ozone, gas (SO_2 and NO*x*), etc., are capable of damaging DNA indirectly (Patra *et al*. 2005).

Chemical mutagenesis is an approach practiced to induce mutations in plants for the improvement of beneficial agronomic traits (Khan *et al*. 2009, Alcantara *et al*. 1995). This breeding approach has benefit over conventional breeding for yield in itself because it increases the possibility of crosses resulting in additive gene action (Reynolds and Trethowan 2007). Mutations are useful tools in studying the nature and functions of genes which form the building blocks and basis of plant growth and development, hence producing raw materials for genetic improvement of economic important crops such as wheat. The main advantage of mutation breeding is the possibility of improving one or more traits without changing the rest of the genotype (Khan *et al*. 2009).

Different mutagenic agents are available and applicable when inducing favorable mutations at high frequency. Many reports on mutagenic lines revealed a significant improvement in crop yield after the induction of mutations. Many of these chemical mutagens possess the clastogenic properties which have effects on plants (Khan *et al*. 2009). In general, most chemical mutagens induced mutations which lead to amino acid changes, thus alter the function of proteins. Another advantage is the mutants that are produced, greatly facilitate the isolation, identification and cloning of genes used in designing crops with improved yield and quality traits (Khan *et al*. 2009). Also, it is possible to clone the genes affected by these induced mutations, using various techniques such as chromosome walking from restriction fragment length polymorphism (RFLP) sites or T-DNA tagging (Katavic *et al*. 1995). Furthermore, the selected mutants can be

developed directly as breeding lines or cultivars. Additionally, mutants formed add value to a collection of genetic markers and may serve as testers during linkage studies (Alcantara *et al*. 1995, Khan *et al*. 2009).

The efficiency of any chemical mutagen is mainly dependent on its concentration. Other variables such as pre-soaking time, duration of treatment and pH of the mutagen solution at which it was prepared has also proved to be important factors. The hydration of seeds through soaking in water is known to facilitate rapid mutagen infusion (Sarma *et al*. 1979).

Many different chemical mutagens have been reported in the past, but they are not equally effective in the inductions of mutations. In the present study four different mutagens were tested namely: sodium azide (SA), Ethylmethanosulfonate (EMS), *N*-Nitroso-*N*-methylurea (NMU) and Maleic hyrazide (MH) as discussed below.

2.4.1. Sodium Azide (SA)

Sodium azide (SA), an *in vivo* metabolite, was found in the 1970s to be highly effective in producing mutations in barley *(Hordeum vulgare* L.) (Rines 1985). However up until 1978, the biological action and pathway of mutation induction by SA was still not well understood (Sarma *et al*. 1979). It was only later found to be the most efficient mutagen in barley and has become the agent of choice for the induction of mutants in this plant due to the ease with which the mutagen is applied to dry and pre-soaked grains (Olsen *et al*. 1993). Barley seeds germinated in the presence of azide (N^-_3) confirmed that this compound acts as point mutagen during DNA replication (Al-Qurainy 2009). Olsen *et al*. (1993) generated base substitution of which 86% were nucleotide transitions and 14% were transversions, with A-T to G-C base pair transitions roughly 3 times more frequent than G-C to A-T transitions. No deletions or mutation hot spots were found. Besides barley, SA is marginally mutagenic in several other organisms e.g. *Escherichia coli*, yeast, and the housefly. However, it fails to increase mutation frequencies in *Arabidopsis*, *Drosophila*, and *Neurospora* and is only weakly mutagenic in mammalian cells (Khan *et al*. 2009, Olsen *et al*. 1993).

SA induces point mutation of bases in the genome of plants through an organic azide metabolite production. The azide metabolite compound enters into the nucleus, interacts with DNA, and

creates a point mutation in the genome. The azidoalanine metabolite was chemically identified in barley and bacteria as an amino acid analogue L-azidoalanine (N_3-CH_2-CH $(NH)_2$-COOH). The production of this metabolite was found to be dependent on the enzyme O-acetylserine sulfhydrylase (E.C. 4.2.99.8). This enzyme catalyses the condensation of azide (N^-_3) or sulfide (S_2^-) with O-acetylserine to produce azidoalanine or L-cysteine respectively (Khan *et al*. 2009, Al-Qurainy 2009).

The conditions for seed treatment with SA and germination are crucial during the application of SA. The relative effectiveness of azide treatments of seeds vary with the pH of the mutagen solution, azide concentration, length of soaking in water prior to azide exposure, plant species, and plant`s cultivar type used (Rines 1985, Khan *et al*. 2009). At low pH value, the quantity of SA that dissociate to hydrazoic acid is theoretically many times greater than at higher pH values. For example there is approximately 19 times more hydrazoic acid at pH 3 than at pH 6, for the same concentration of SA (Khan *et al*. 2009). Al-Qurainy *et al*. (2009) also reported that SA is highly soluble in water, whilst diluted in phosphate buffer of pH 3.2, it gives a larger number of hydrazoic ions which penetrate into the cell and create mutations. SA is found to be non-effective when dissolved in base solutions, but it is a strong mutagen when dissolved in acidic solutions.

Mutation frequency, as well as the biological damage shows a linear response with the increase in the concentration of azide. It was found that azide induce maximum mutations in rice seeds pre-soaked in water for longer durations, however, excessive longer soaking periods adversely affected the azide mutagenicity with extreme periods of soaking yielding fewer mutation frequencies (Sarma *et al*. 1979).

2.4.2. Ethylmethanosulfonate (EMS)

Ethylmethanosulfonate (EMS) is a monofunctional alkylating clastogen known to induce chemical modification of nucleotides through depurination which results in DNA strand breaks and lesions (Patra *et al*. 2005). EMS treatment has a strong biased alkylation of guanine (G) residues forming O^6- ethylguanine, which can pair with thymine (T) but not with cytosine (C) (Kim *et al*. 2006). A study done in *Arabidopsis* indicated that EMS mutagenesis generated

randomly distributed mutations throughout its genome, suggesting the potential of changing loci of particular interest without inducing a great number of closely linked mutations. This allows a plant breeder to obtain useful alleles without the cloud of linked deleterious alleles present in exotic or wild germplasm or even from adapted inbred lines (Kim *et al*. 2006).

EMS like azide treatments has also been reported to yield fewer detectable chromosomal aberrations (Rines 1985). If these chromosomal breaks lead to losses of large segments of chromatin and consequently lose multiple loci, then such mutations would be more likely to show phenotypic expression than simple point mutations (Rines 1985).

Seed treatments with EMS have shown that the frequencies of mutations are dependent on the location of the genes in the genome and the treatment conditions during mutagenesis. The most important parameters for inducing mutations with EMS are concentration, duration of treatment and solution temperature (Alcantara *et al*. 1995). Previous studies suggested the mutation frequency to increase proportionally with increasing EMS concentration (Hsie *et al*. 1975) and longer exposure times (Alcantara *et al*. 1995). This implies that higher concentrations and longer seed exposure time favors the induction of significant mutations at higher accuracy, thus emphasizing the importance of concentration exposure time of seed to EMS to induce even greater number of mutants. However, at higher concentrations (more than 0.9 %) EMS resulted in a reduced frequency of induced mutations in soybeans (Kim *et al*. 2006).

2.4.3. *N*-Nitroso-*N*-methylurea (NMU)

N-Nitroso-*N*-methylurea (NMU) is a highly reactive alkylating agent (Rank and Nielsen 1997), which exhibits its toxicity by transferring its methyl group to nucleobases in nucleic acids and thus inducing mutations (Juchimiuk *et al*. 2007). It is also a highly reliable carcinogen (Rank and Nielsen 1997) that has been shown to act as a universal carcinogen, and as placental carcinogen in experimental animals. NMU may be of particular importance in humans, since it can be formed *in vivo* by nitrite ions reacting with urea derivatives in the gastrointestinal tract. Furthermore, it has been shown to be mutagenic in *Aspergillus nidulans* and in *Escherichia coli* (Sanger and Eisen 1976).

In a study where human fibroblast cell lines were pulse-treated for 1 h with NMU at various time intervals before conducting chromosome analysis, an increase in chromatid-type abnormalities was reported. Cells were treated with 1 X 10^{-3} M, 5 X 10^{-4} M, and 1 X 10^{-4} M of NMU during the M or G_2 phases of the cell cycle, and the results showed a substantial increase in abnormalities when compared with the controls (Sanger and Eisen 1976). The mitotic indices and occurrences of abnormalities proposed a concentration response effect when cells were treated with the two highest concentrations, i.e. 1 X 10^{-3} M and 5 X 10^{-4} M. From these findings, Sanger and Eisen (1976) concluded that NMU treatment of human diploid cell lines *in vitro* induced both chromosome and chromatid aberrations.

The concentration of NMU plays a pivotal role in its toxicity and effectiveness (Alghamdi *et al*. 2010). Laboratory studies conducted to estimate the critical concentration of NMU on seedling emergence and growth of different barley varieties namely viz, Acsad-176 and Rum (Alghamdi *et al*. 2010), used NMU concentrations of 0.5 mM, 1.0 mM, 1.5 mM, 2.0 mM for 3 hours, respectively and showed that higher treatments concentrations substantively reduced shoot and root growth. The effects where more evident on roots than shoots. The outcomes of this study indicated that a concentration of 2.0 mM NMU was toxic to the analyzed varieties, and recommended lower concentrations for field experiments to avoid increased levels of mutated phenotypes in barley fields (Alghamdi *et al*. 2010).

In canola (*Brassica napus* L.), higher NMU concentrations and longer treatment durations, resulted in a reduction of germination rate, germination percentage, and seedling height (Emrani *et al*. 2011). Different NMU concentrations (3, 6, 9 and 12 mM) were used with each concentration treatment conducted for 2, 4, 6 and 8 hours. In the experiment it was found that a two hour treatment of non-presoaked seeds with 3 mM NMU led to the highest radical length and seedling height, while, non-presoaked seeds treated with 12 mM NMU for eight hours produced the lowest values for all the measured traits (i.e. germination percentage, germination rate index and the seedling height) (Emrani *et al*. 2011).

2.4.4. Maleic Hydrazide (MH)

Introduced in 1950 (Swietlinska and Zuk 1978) maleic hydrazide (1, 2 dihydro-pyridazine-3, 6-dione) (MH) is a structural isomer of the pyrimidine base uracil (Gichner *et al*. 2000, Cortes *et al*. 1985, Swietlinska and Zuk 1978, Ponnampalam *et al*. 1983, Marcano *et al*. 2004), originally synthesized through reacting hydrazine sulfate with maleic anhydride in aqueous NaOH medium (Ponnampalam *et al*. 1983). MH has a global use as herbicide, fungicide and/or growth regulator in agriculture with selective sensitivity to the toxic effects of MH well documented in higher plants (Gichner *et al*. 2000, Cortes *et al*. 1985, Swietlinska and Zuk 1978, Ponnampalam *et al*. 1983, Marcano *et al*. 2004). This phenomenon can be explained due to the fact that various plant species express different abilities to detoxicate different chemicals (Swietlinska and Zuk 1978).

MH is well-known to be an active chromosome-breaking agent in higher plants (Swietlinska and Zuk 1978). The chromosome-breaking effect of MH on plant chromosomes corresponds closely to the chromosome-breaking properties of other alkylating agents such as EMS (Swietlinska and Zuk 1978). Based on its crystal structure and the probable formation of base pairs with nucleic acid bases, suggests that MH, by incorporating into RNA, may interfere with the incorporation of any nucleosides into this molecule (Swietlinska and Zuk 1978, Ponnampalam *et al*. 1983). Besides these findings, MH has been studied for its cytogenetic action on animals and plants (Cortes *et al*. 1985, Marcano *et al*. 2004, Swietlinska and Zuk 1978, Ponnampalam *et al*. 1983).

In mammals, MH shows low toxicity, however in some instances. Ponnampalam *et al*. 1983 reported a decrease fertility in rats after feeding on potatoes containing MH residues. The chemical was also reported to cause chromosomal breakages in mice (Swietlinska and Zuk 1978, Ponnampalam *et al*. 1983). Although several studies documented an induction of cytogenetic effects in various groups of mammals, only a few chromosomal aberrations in mammalian cells have been reported (Gichner *et al*. 2000, Swietlinska and Zuk 1978, Ponnampalam *et al*. 1983, Cortes *et al*. 1985). However, MH-induced chromosomal aberrations have been documented in grasshoppers and in fish (Swietlinska and Zuk 1978).

pH is another factor that influences the functionality of the MH compound. It has been demonstrated that the effect of MH increases when the pH is low (Cortes *et al*. 1985,

Ponnampalam *et al*. 1983), for example that at pH 4 the chromosome breaking ability of MH is higher than at pH 6 (Gichner *et al*. 2006).

2.5. The identification of induced mutations

2.5.1. Amplified fragment length polymorphism (AFLP)

Amplified fragment length polymorphism (AFLP) is a PCR-based tool used by molecular biology researchers, and is also applicable in genetic engineering practices and DNA fingerprinting. AFLP was developed using restriction enzymes to digest genomic DNA which is followed by ligation of adaptors to the sticky ends of the restriction fragments. A subset of the restriction fragments is then selected to be amplified. This selection is achieved by using primers complementary to the adaptor sequence, the restriction site sequence and a few nucleotides inside the restriction site fragments (Vos *et al*. 1995).

The amplified fragments are visualized on denaturing polyacrylamide gels either through fluorescence or autoradiography techniques. AFLP-PCR is a highly sensitive and reproducible technique for distinguishing polymorphisms in DNA including induced mutations. The AFLP technology has more benefits over other marker systems because of its ability to identify various polymorphisms in different genomic regions while amplifying between 50 and 100 fragments in one time with no prior sequence information needed for the amplification step. As a result, AFLP has become widely used for the identification of genetic variation in strains or closely related species of plants, fungi, animals, and bacteria (Zabeau and Vos 1993). In the present study, AFLPs were applied to identify genomic regions that have been modified after being treated with a mutagen.

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Chapter 3: Development of drought tolerance in wheat (*Triticum aestivum* L.) using induced mutagenesis

3.1. Introduction

Mutagenic or mutation breeding technology started in the early 1930s using mainly X-rays as a preferred form of mutagen (Hassan *et al*. 2012, Khan *et al*. 2005). Mutation breeding serves as an alternative approach to develop improved crop cultivars in plant breeding through the incorporation of genetic changes and the formation of new genetic resources (Hassan *et al*. 2012, Khan *et al*. 2005). Induced mutations have been used mainly to generate variations that are naturally rare or could seldom be found in germplasm collections (Emrani *et al*. 2011) as well as crops with low genetic variability and those that are not amenable to improvement through conventional breeding methods (Hassan *et al*. 2012).

Chemical mutagenesis in particular has a number of advantages over physical mutagenesis such as the capacity to use different mutagens, change mutagen concentrations and the ease to scale the size of the mutagenesis procedure. Optimization of mutation induction conditions in plant species plays a key role in the effective application of the mutagenic events (Emrani *et al*. 2011). Among the chemical mutagens, ethyl methane sulphonate (EMS), diethyl sulphate (DES), methyl nitroso urea (MNH), ethyl nitroso urea (ENH), ethyleneimine (ED), nitroso- N-methylurea (NMU) and N-Nitroso, N-Ethylurea (ENU) are among the most commonly used mutagens, all of which belong to a special class of alkylating agents (Alghamdi *et al*. 2010, Emrani *et al*. 2011). Alkylating agents add ethyl or methyl groups to bases in the nucleotide structure, which could potentially lead to silencing an active gene, activating a silent gene, or altering a particular gene action with a vast usage in forward and reverse genetic screening (Emrani *et al*. 2011). Most of these alkylating agents used during induced mutagenesis have been found to be more effective than physical mutagens such as radiations (Juchimiuk *et al*. 2007). As a non-alkylating agent sodium azide (SA) is sometimes preferred in plant mutation induction, because it mainly creates point mutations (Emrani *et al*. 2011).

A large number of plant varieties have been developed with improved traits using mutational breeding and included to date 2,570 mutant varieties that have been officially released worldwide (Hassan *et al*. 2012). Although induced mutagenesis for crop improvement had been applied since the 1950`s, research on induced mutagenesis in breeding is still an active and ongoing activity in many countries, for instance, the mutation breeding program in durum wheat (*T.*

durum) in Italy began in 1956 and yielded around 1,000 mutants, 292 of which are considered useful for breeding purposes. Six were released as new varieties, while a further five varieties were produced by selection and hybridization among mutants (Alghamdi *et al*. 2010). Chemical induced mutagenesis applied in the former USSR since 1965, resulted in the development of 499 mutant crop varieties (Alghamdi *et al*. 2010). Mutant varieties of winter wheat, spring and winter barley and maize account for over 25% of these released varieties. In Pakistan, wheat 'Soghat 90' and 'Kiran 95', both derived using sodium azide treatment, express high grain and biomass yield, high protein and lysine content and tolerance to leaf rust (Alghamdi *et al*. 2010).

The objective of this study was to develop superior wheat lines with improved drought tolerance using chemical induced mutagenesis. This was achieved through the application of recommended concentrations of mutagens and phenotypically screening procedures in order to score for drought tolerant plants.

3.2. Materials and methods

3.2.1. Seed material, chemical mutagens and experimental solutions

'TugelaDN' (Tugela/SA1684*7) seeds available in the laboratory was used for the current investigation as breeding material. The chemical mutagens used in the mutagenesis experiments included: sodium azide (SA); ethyl methanesulfonate (EMS); maleic hydrazide (MH) (all obtained from Merck, Germany); and *N*-methyl-*N*-nitrosourea (MNU) acquired from Sigma, (South Africa). To avoid rapid hydrolysis or dissociation of mutagens, all mutagens were prepared by dissolving in a 0.1 M potassium phosphate buffer (pH 7.5) (Alcantara *et al*. 1995, Rines 1985, Sarma *et al*. 1979, Roychowdhury and Tah 2011). Thereafter the solutions were filter sterilized using sterilized filters (0.22 µm) and then stored at 4^{o}C until further use (Al-Qurainy 2009). The 0.1 M phosphate buffer was also used for dilution of the chemical mutagens (Kim *et al*. 2006). For the negative and positive controls distilled water (dH_2O) was used. Positive controls received normal watering while negative controls were exposed to similar water stress conditions as with the chemical mutagen treatments during drought stress by not providing enough water unlike in the positive controls.

3.2.2. Chemical mutagen treatments

A total of 250 seeds per treatment were pre-soaked in a 50 ml plastic test tube containing 30 ml of 0.1 M phosphate buffer (pH 7.5) at 4^{o}C for 16h (Olsen *et al*. 1993, Lee *et al*. 2011, Kim *et al*. 2006). Thereafter the buffer was decanted and fresh 0.1 M phosphate buffer (pH 7.5) was added where after seeds were soaked with aeration on a shaker at room temperature for 8h. After the soaking step, seeds were treated in oxygenated solutions of either SA (0.1 mM, 1 mM and 10 mM), EMS (0.1%, 0.5%, 1.0% v/v), NMU (0.5 mM, 1.0 mM, 2.0 mM), MH (0.5 mM, 1.0 mM and 2.0 mM). Seeds were exposed to each mutagen concentration for 2h, 4h and 8h, respectively. With the controls, the seeds were treated under similar conditions as with the mutagen treatments but using oxygenated water. Intermittent shaking was done to provide uniform treatment to the dipped seeds (Roychowdhury and Tah 2011) and low temperatures (less than room temperature) were applied to maintain minimal metabolic activity of the seeds (Sarma *et al*. 1979). After each treatment, seeds were rinsed 3 to 4 times using distilled water that has been autoclaved in order

to remove excess mutagens (Al-Qurainy 2009). The seeds were finally spread on paper in order to dry (Rines 1985). The treatments were conducted independently during February 2012 for SA, March 2012 for EMS, August 2012 for NMU and September 2012 for MH.

3.2.3. Planting mutagenized (M_0) seeds and growth conditions

After the treatments with chemical mutagens, seeds were sown into plastic trays (15.5 cm X 30 cm X 10 cm) containing compost soil mix as described by Lee *et al*. (2011). After sowing, the seeds were covered with 2 cm layer of compost soil (Alghamdi *et al*. 2010, Juchimiuk *et al*. 2007), watered and placed in the green house under controlled conditions (19-24°C) for direct screening of putative drought tolerant and dominant mutants. Treatment and experiments were carried out at the Welgevallen experimental farm (Stellenbosch University) (-33.942635, 18.866424) (Figure 3.2.3). After planting, the mutagenic parents (M_1) were left to yield mutagenic progeny seeds for further screening for recessive as well as dominant mutants.

Figure 3.2.3: Welgevallen experimental farm (Stellenbosch University).

3.2.4. Days to germination and germination rate

Days to seed germination (Table 3.3.1) were recorded as soon as there was shoot emergence. Germination rate (G %) was calculated using a modified equation from Melki and Marouani (2009), as follows:

$$G\ (\%) = \frac{\text{Number of germinated seeds}}{\text{Total number of seeds}}\ X\ 100 \qquad \text{(Equation 3.2.4)}$$

3.2.5. Drought stress, screening procedures and selection of putative drought tolerant lines

After 2 to 3 weeks of visually evaluating seedling emergence, water supply was terminated in all treatments and negative controls, while positive controls received normal watering of approximately 1000 ml per tray until soil was wet. Using a modified gravimetric formula from Black (1965) the water moisture content during and after drought stress was monitored, calculated and recorded (Table 3.3.2)

For example:

Mass Beaker (M_B) = 18.54g

$M_B + M_{WS1}$ = 23.54g (M_{WS1}: Mass wet soil before heating). This weight is placed in an oven for approximately 24 hours

24h ($M_B + M_{ws2}$) = 21.72g (M_{WS2} Mass wet soil after heating)

Moisture content = $M_{WS1} – M_{WS2}$

Moisture content = 23.54 – 21.72 = 1.82 ml of H_2O

A TFA Garden Combitester (Qualitäts-Erzeugnis Germany) was also used to access moisture content in the soil following the manufactures guide. In brief, two probes were inserted into the soil between the edge and the center of the tray to about 3/4 of the length of the probe. The

reading was taken from the center scale and a comparison was made with the optimum moisture level specified to the plants as suggested in the manual guide table provided. This instrument was also used to monitor pH of the soil.

After 2 to 3 weeks, the plants that survived the induced water stress were considered to be putative water stress tolerant mutants. Selected plants were transferred into pots and grown under normal recommended cultural practices and plant protection measures were followed timely to raise a good matured crop stand (M_1). The number of plants that survived till maturity including the time of flowering phase and harvesting, were scored from each treatment and recorded as percentage survival (S%) using the following formula:

$$\text{Percentage survival } S\,(\%) = \frac{\text{Number of plants that reached maturity}}{\text{Total number of initial plants planted}}\ X\ 100 \qquad \text{(Equation 3.2.5)}$$

Plants considered as putative water stress tolerant were scored on a scale of 1-3 for their phenotypic symptoms such as wilting (turgidity), chlorosis and leaf rolling (Small 2012); where in all cases plant`s scoring 1: Represented healthy plants, 2: Intermediate state and 3: Representing drought sensitive plants (Figure 3.2.5A and Figure 3.2.5B).

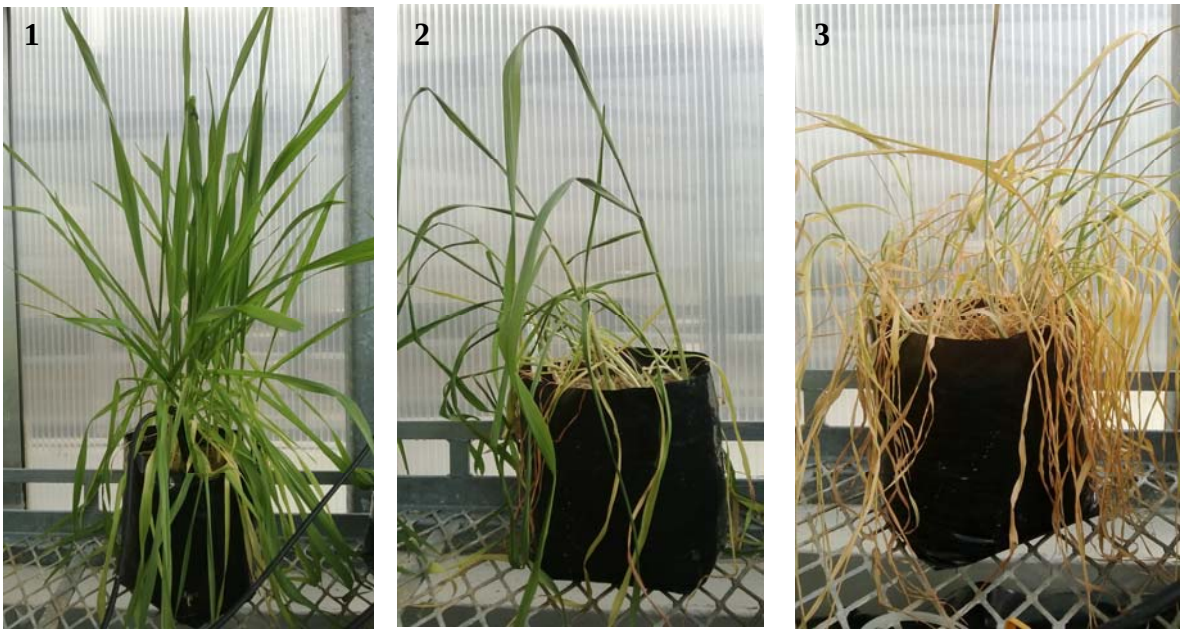

Figure 3.2.5A: Plant phenotype for scoring turgidity where **1** = Healthy plants with no wilting (tolerant plants); **2** = Partial wilting (intermediate tolerant plants); **3** = Complete wilting or death (drought sensitive plants).

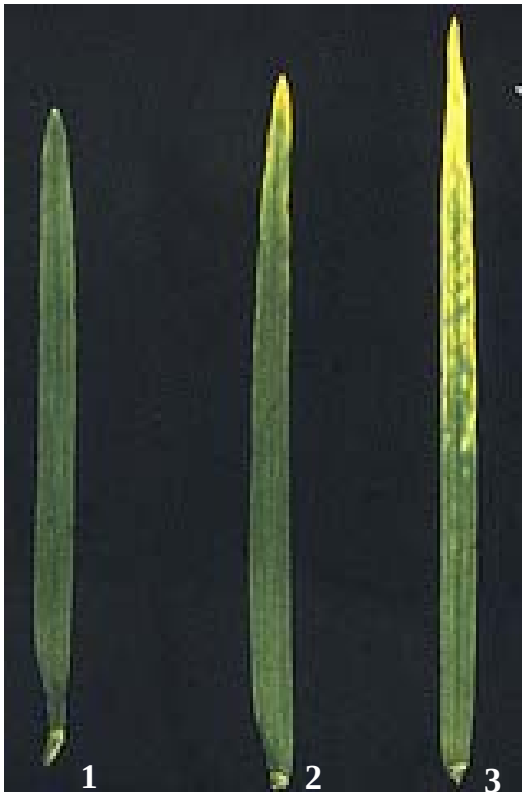

Figure 3.2.5B: Plant`s phenotype for the scoring of leaf chlorosis as a measurement of tolerance or susceptibility where **1** = healthy plants with no chlorosis; **2** = Partial chlorosis (intermediate tolerant) and **3** = Complete chlorosis or death (drought sensitive plants). (Image obtained from CIMMYT, 2006).

Using a ruler, plant height (cm) was also measured throughout the plant life cycle. Final plant height was defined at maturity by measuring the highest shoot.

In the established M_1 generation the following data were also recorded:

- Morphological development of plants.
- The date when lines reach two main developmental stages (i.e., anthesis and maturity), days to flowering, number of flower/plant and number of leaves/plant.
- Seed production.

3.2.6. M_2 and M_3 generations

Seeds harvested from the putative drought tolerant mutant plants (M_1) were planted to produce M_2. The M_2 was not screened for drought tolerance and were watered at regular intervals to produce seeds for germplasm collection and future field trials. Seeds collected from M_2 were also planted to produce M_3 that were screened to confirm drought tolerance using similar conditions as applied during screening M_1. Leaf material was collected from each generation for genomic DNA extraction to be used during molecular characterization. Traits that were studied in both M_2 and M_3 included; days to germination, germination rate, height, general plant development and seed production qualities such as seed count, seed morphology and seed mass. These qualities were compared to the parents and controls in respective generations.

3.3. Results

3.3.1. Days to seedling germination and seed germination rate

After sowing seeds, seedling emergence was compared between the various concentrations and duration of treatments for all the mutagens. Seed germination was scored based on the appearance of the plumule, cotyledons and radicle as evidence of these structures implied the final stage of seed germination (Alcantara *et al*. 1995, Bewley and Black 1985). Germination rates were determined with a subset of 2,250 seeds planted from each treatment and compared to the control. SA, NMU and MH treatments took approximately four days to germinate irrespective of their concentrations or treatment duration. These results were similar to that observed in the controls. EMS showed a different trend whereby there was a noticeable effect on seedling germination as well as seed germination rate depending upon the EMS concentration and length of treatment (Table 3.3.1). EMS (0.5% and 1.0%) treatments took longer to germinate with approximately seven days while 1% EMS 8 h deviate the most with an average germination of about twelve days.

Table 3.3.1: The effect of mutagen concentration and treatment period on days to seedling germination

Mutagen	Concentration (mM / %)	Days to seeding germination (days) (Treatment period (h))		
		2	4	8
SA	0.1 mM	4±1	3 ±1	4±1
	1.0 mM	3±1	4±1	3±1
	10 mM	4±1	4±1	4±1
EMS	0.1%	4±1	4±1	4±1
	0.5%	4±1	7±1	7±1
	1.0%	7±1	7±1	12±1
NMU	0.5 mM	4±1	4±1	4±1
	1.0 mM	4±1	4±1	4±1
	2.0 mM	4±1	4±1	4±1
MH	0.5 mM	4±1	4±1	4±1
	1.0 mM	4±1	4±1	4±1
	2.0 mM	4±1	4±1	4±1
Control	nt	4±1	4±1	4±1

nt= not treated

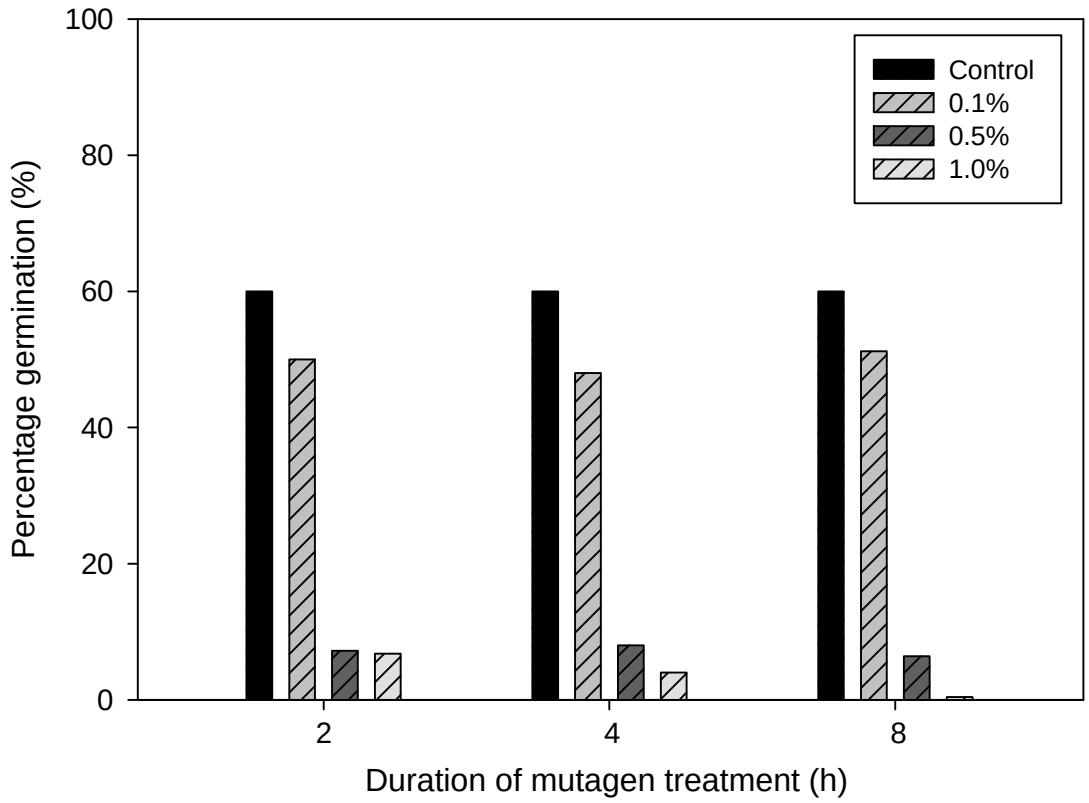

Figure 3.3.1A: Seedling germination rate across EMS treatments at different concentrations and treatment duration.

EMS concentration of 0.1% regardless of treatment duration resulted in the highest germination rate of approximately 50%, while 0.5% and 1.0% yielded much lower germination rates (less than 10%) (Figure 3.3.1A). Similar to 0.1% treatments, the duration of treatment with 0.5% EMS had no effect on the germination rate, while longer treatment durations with 1% EMS had dire consequences on germination rates. The lowest relative number of germinating seeds and the largest delay in germination was observed in plants after a 1% EMS 8h treatment in which after twelve days less than 5% of treated plants germinated. The proportion of plants showing lower germination rate is a primary indication of damage caused by the EMS treatment (Roychowdhury and Tah 2011). The results of the study on EMS indicated an overall reduced germination percentage with increasing mutagen concentration and treatment periods.

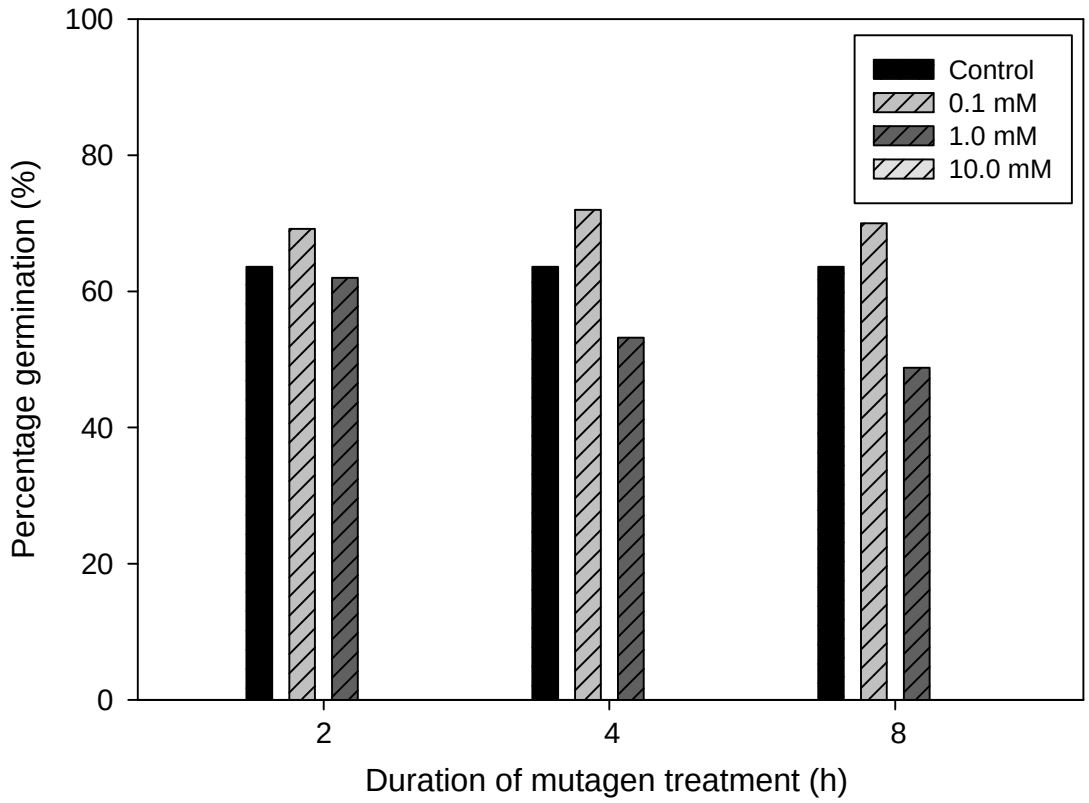

Figure 3.3.1B: Seedling germination rate across SA treatments at different concentrations and treatment duration.

Like EMS, to analyze the SA concentration and treatment duration effect in more detail, wheat seeds were treated with different concentrations of SA (0.1 mM, 1.0 mM and 10 mM) at 2h, 4h and 8h for each concentration. There was no significant difference in germination rates for the 0.1 mM across all treatment exposure times with germination rates of approximately 70% uniformly distributed across all treatments. Like EMS treatments, the germination rate in the SA treatments declined with longer exposure times. This is evident whereby at 1.0 mM, with the 2h treatment duration, over 60% germination was displayed while there was a slight decrease in germination observed at 4h and 8h having lower germination rates of approximately 50% and 48% respectively, while there was 0% germination in all 10 mM SA treatments.

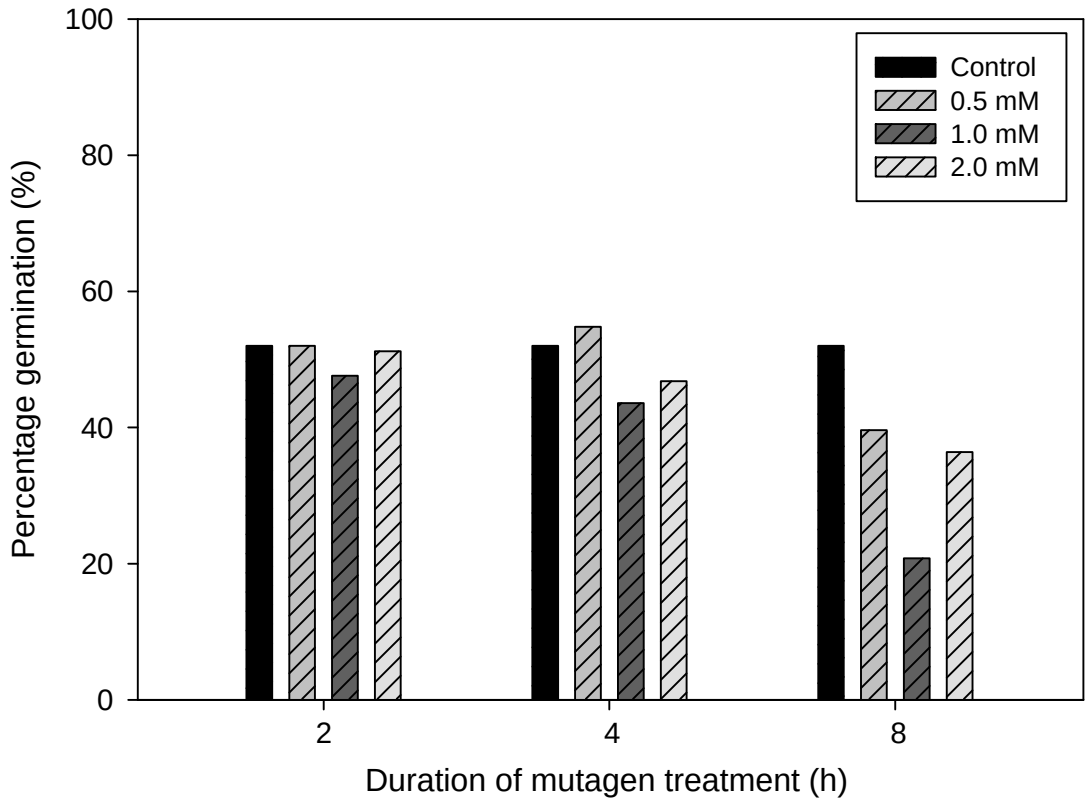

Figure 3.3.1C: Seedling germination rate across NMU treatments at different concentrations and treatment duration.

Unlike EMS and SA treatments, the lowest and the highest NMU concentrations (i.e. 0.5 mM and 2.0 mM) displayed slightly higher germination rates than the intermediate concentration of 1.0 mM in all the treatment durations conducted (Figure 3.3.1C). However the 8h treatment duration in all concentrations of NMU displayed lower germination rate, while was there no significant difference in germination rate between the 2h and 4h treatments durations.

Unlike NMU, the MH treatments with lowest concentrations (0.5 mM and 1.0 mM) displayed slightly higher (though not significant) germination rates than the highest 2.0 mM concentration (Figure 3.3.1D), with treatment duration not having significant effect on germination rates.

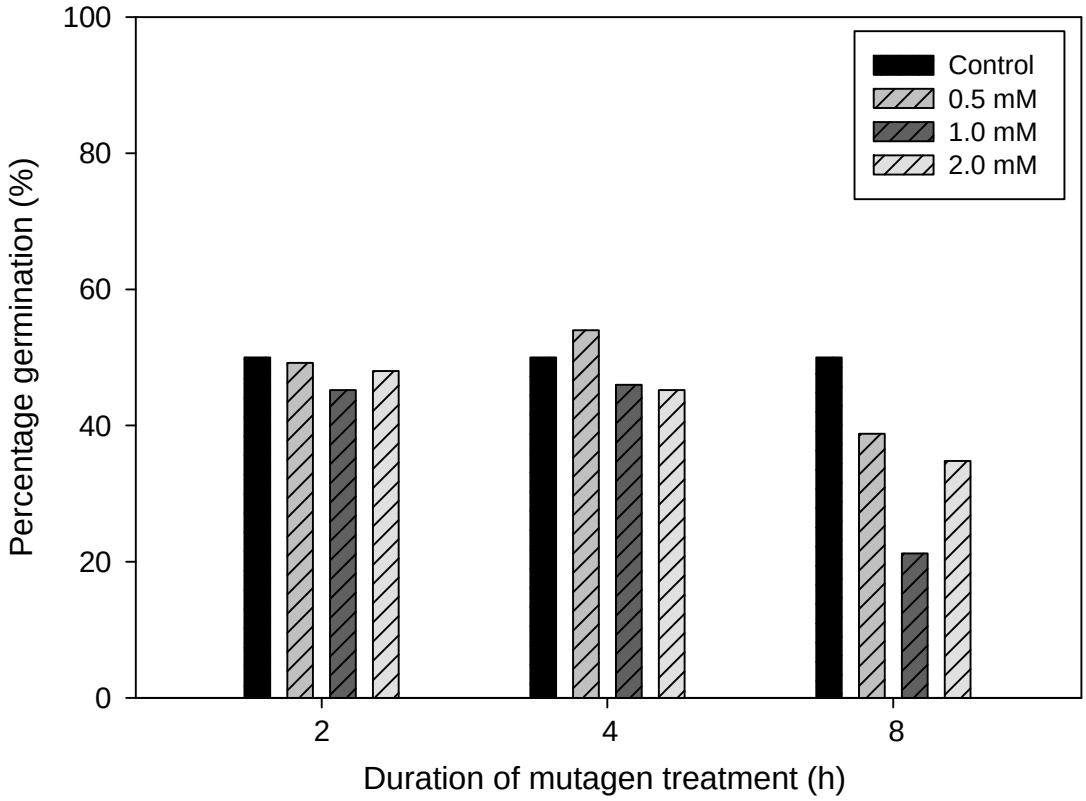

Figure 3.3.1D: Seedling germination rate across MH treatments at different concentrations and treatment duration.

3.3.2. Water stress and putative mutant survival rate

Table 3.3.2: The average moisture content before and after drought stress across all treatments (M_1).

Mutagen	Water content before stress (ml)	Water content after stress (ml)	Percentage water loss (%)
EMS	1.98±0.5	0.11±0.5	94±0.5
SA	2.01±0.5	0.18±0.5	91±0.5
NMU	2.00±0.5	0.23±0.5	86±0.5
MH	1.50±0.5	0.15±0.5	90±0.5

To select for mutants that express water stress tolerance, plants were exposed to water stress. All the mutagen treatments and one set of untreated plants (negative control) were not watered until the negative control died. While the positive control (untreated plants) were watered on a regular basis. When exposed to stress conditions with excessive water loss (Table 3.3.2), untreated negative controls, NMU and MH treatments demonstrated zero survival rates. On the other hand, a few of the plants treated with SA (Figure 3.3.2B) and EMS (Figure 3.3.2C) survived. The plants that survived the stress treatment were transferred into pots where all normal recommended cultural practices and plant protection measures were followed to raise good crop stand.

Figure 3.3.2A: Phenotypic confirmation of water stress tolerance in the selected M_1 mutant line. 1 mM SA 2h treatment and negative control plants responding to drought stress when both were exposed to similar water stress conditions.

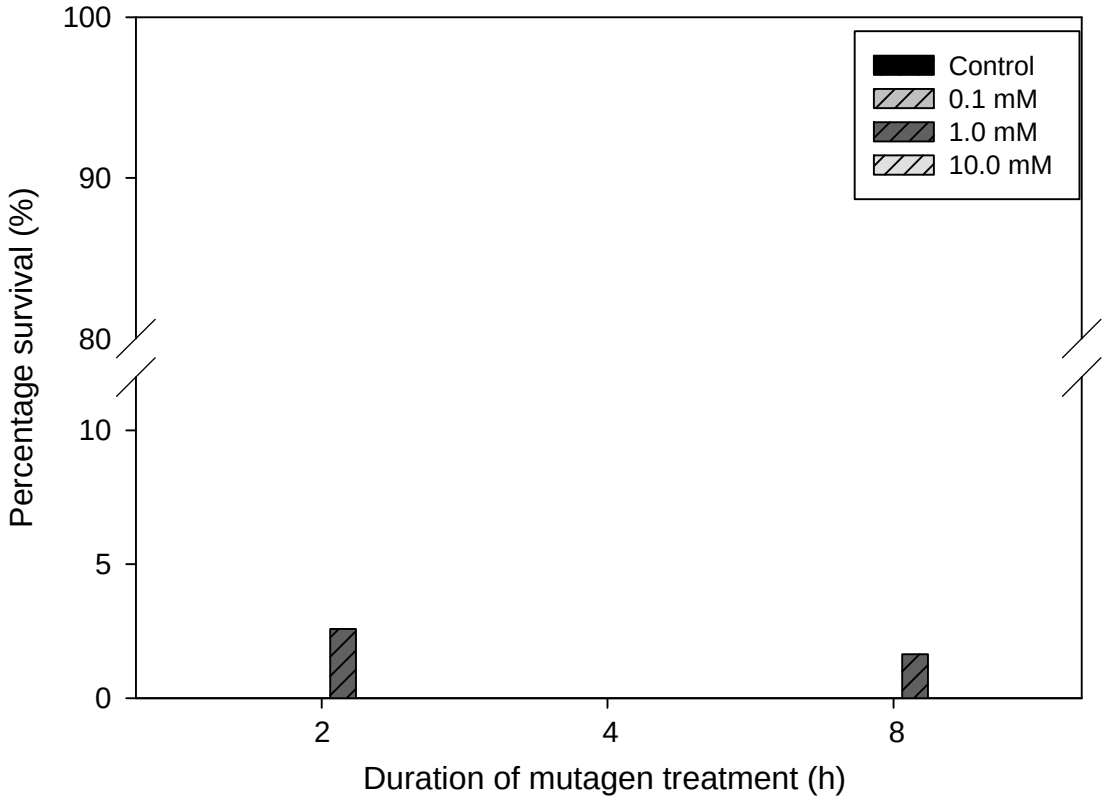

Figure 3.3.2B: Survival rates of SA treatments compared to control.

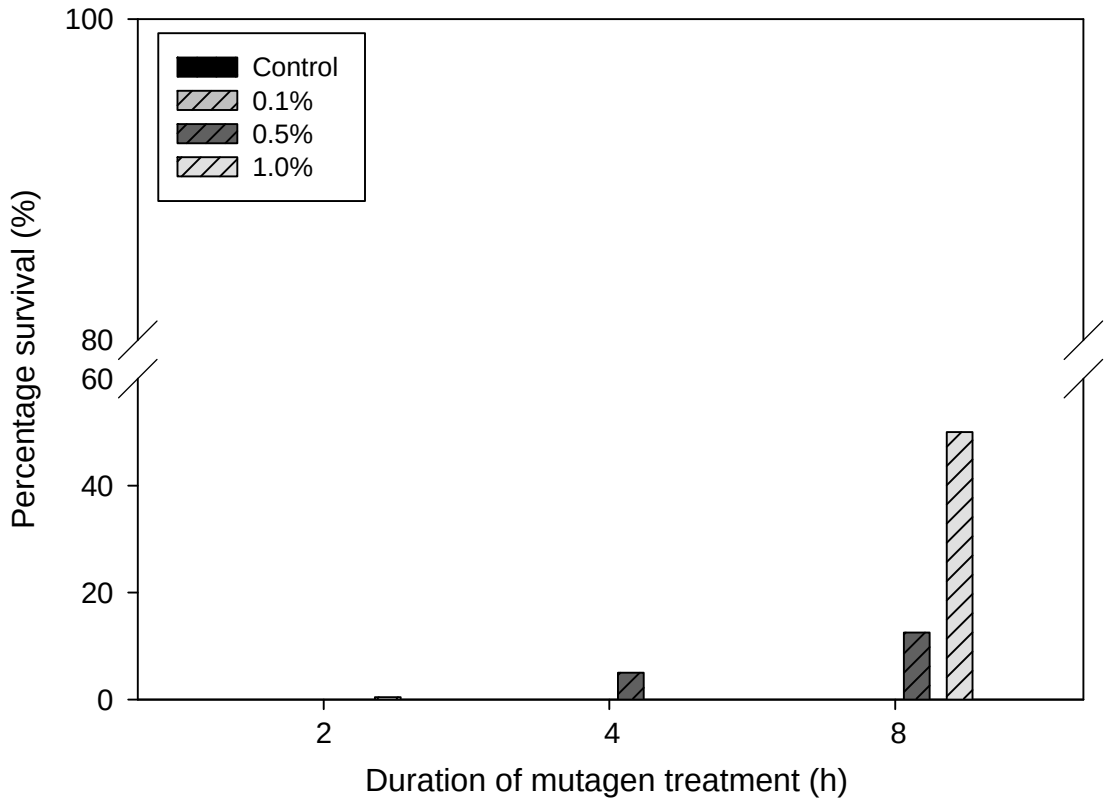

Figure 3.3.2C: Survival rates of EMS treatments compared to control.

3.3.3. Plant development and putative mutant plants heights

A considerable reduction in plant height was found in the treated SA and EMS plants with these plants reaching only the height of approximately 48 cm upon maturity.

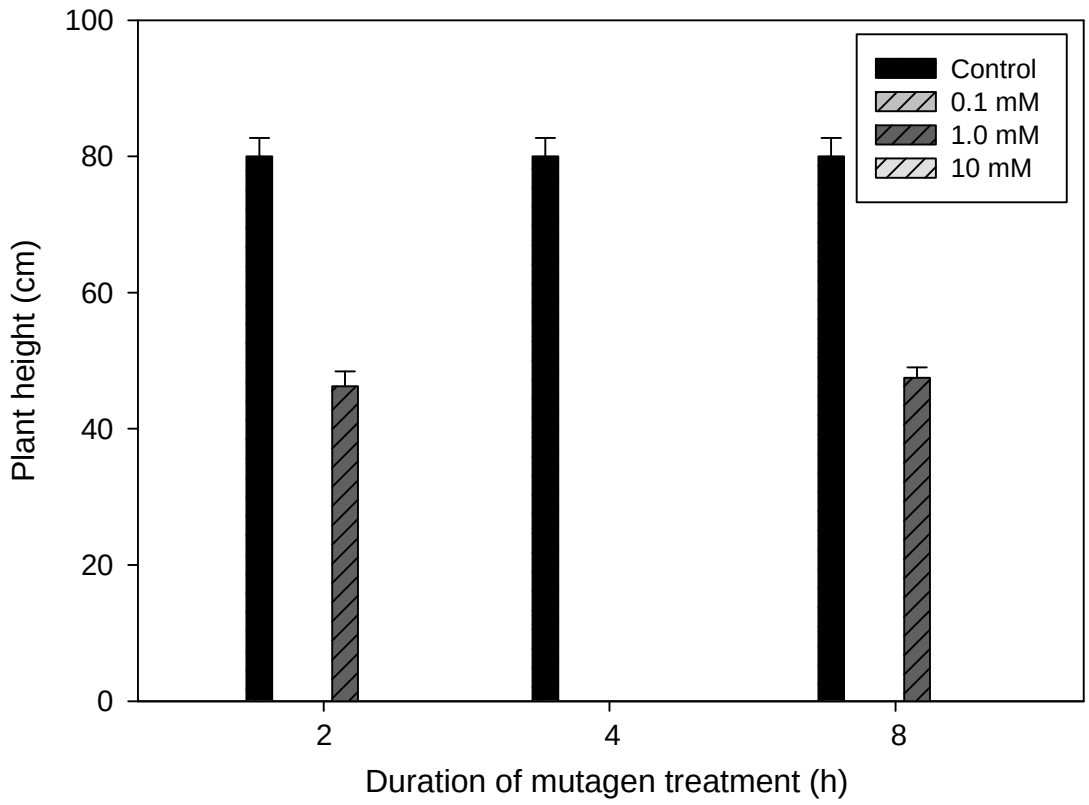

Figure 3.3.3A: Average height of SA treated putative mutants compared with the positive controls.

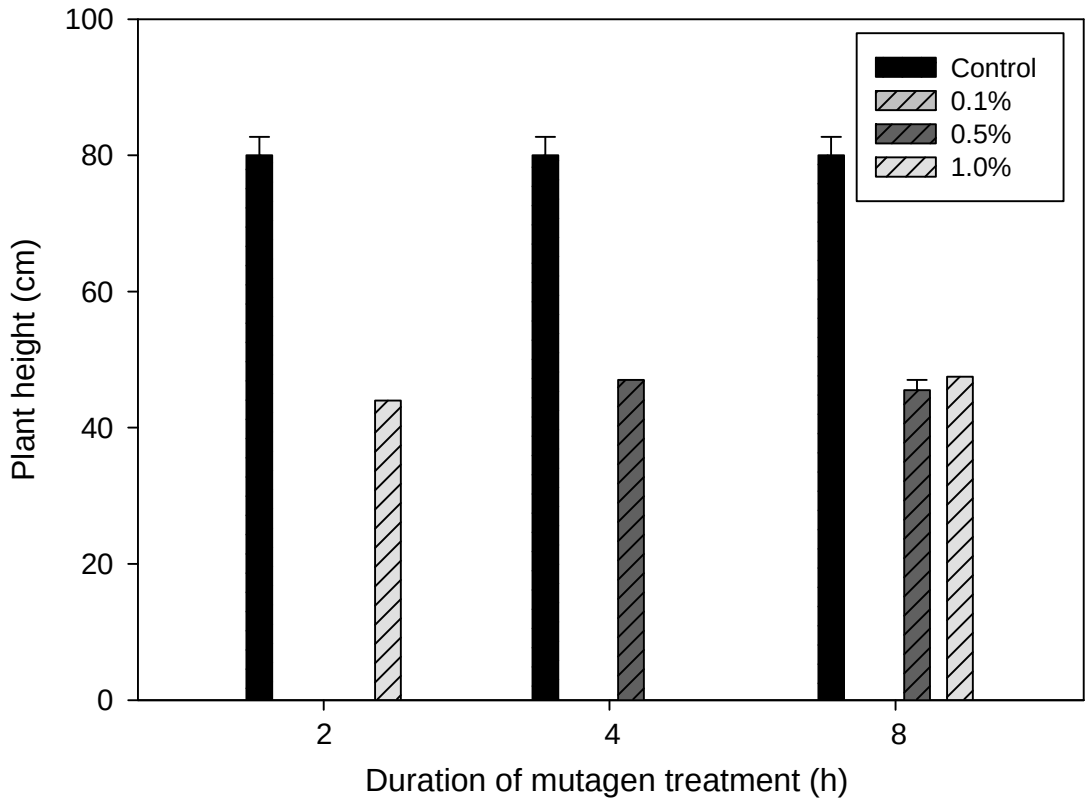

Figure 3.3.3B: Average height of EMS treated putative mutants compared with the positive controls.

Only one mutant was derived from 0.1% (2h), 0.5% (4h) and 1.0% (8h) EMS treatments and thus no standard deviation was determined.

Figure 3.3.3C: Treated plant grown to a good matured crop stand but expressed stunted growth compared to control.

3.3.4. Flowering, maturation and seed production.

Table 3.3.4: Effect of the different treatments on days to flowering, number of seeds produced and average seed weight.

Accession number*	Treatment	Days to flowering	Number of seeds	Thousand Kernel Weight (TKW) (g)
SU/2012/SA/1/2/10	SA 1 mM (2h) 10	-	0	-
SU/2012/SA/1/2/16	SA 1 mM (2h) 16	290±10	375	0.0297
SU/2012/SA/1/2/6	SA 1 mM (2h) 6	-	0	-
SU/2012/SA/1/2/21	SA 1 mM (2h) 21	-	0	-
SU/2012/SA/1/8/21	SA 1 mM (8h) 21	300±10	256	0.0273
SU/2012/SA/1/8/1	SA 1 mM (8h) 1	-	0	-
SU/2012/SA/1/8/16	SA 1 mM (8h) 16	-	0	-
SU/2012/EMS/0.5/4/7	EMS 0.5% (4h) 7	230±10	260	0.0298
SU/2012/EMS/0.5/8/6	EMS 0.5% (8h) 6	200±10	0	-
SU/2012/EMS/0.5/8/11	EMS 0.5% (8h) 11	-	0	-
SU/2012/EMS/1/2/12	EMS 1% (2h) 12	223±10	266	0.0281
SU/2012/EMS/1/8/1	EMS 1% (8h) 1	210±10	0	-
Control	nt	100±110	1500	0.0351

- = growth or sterile plants limited.

nt = not treated.

*Accessions are denoted as follows: Institution identity/ year when experiments were conducted/ chemical mutagen applied/ mutagen concentration/ treatment duration/ plant number.

Putative mutant wheat plants also showed slower growth rates and delayed maturity. In the M_1 generation observations on days to flowering, number of flowers/plants, were also noted. Not all the mutant treated plants reached maturity and flowered. Some of the putative mutants were completely sterile and could not be used for further studies because no seeds were produced as indicated on Table 3.3.4. The fertile mutants generated in this study were harvested for further fingerprinting (Chapter 4). Table 3.3.4 summaries the results on days to flowering, days to maturity, number of seeds produced and Thousand Kernel Weight (TKW) of M_1

Of the seven SA-derived mutants only two produced seed (Table 3.3.4). Both expressed a delayed flowering of 290 (SU2012/SA/1/2/16) and 300 (SU2012/SA/1/8/21) days respectively. Accession SU2012/SA/1/2/16 produced 375 seeds, while mutant accession SU2012/SA/1/8/21 produced 256 seeds. The mutant seed displayed a wrinkled morphology and lower average seed mass when compared to control (Figure 3.3.4).

Of the five EMS derived mutants four flowered (i.e. SU2012/EMS/0.5/4/7, SU2012/EMS/0.5/8/6, SU2012/EMS/1/2/12, and SU2012/EMS/1/8/1). However only two of these produced seed i.e. plant accession number SU2012/EMS/0.5/4/7 and SU2012/EMS/1/2/12. As in the case with the SA mutant plants, these produced seeds were also wrinkled in appearance (Figure 3.3.4) with lower seed mass (Table 3.3.4).

Control SA 1mM (2h) EMS 1% (2h)

Figure 3.3.4: Comparison in seed morphology between treatments and control.

3.3.5. M_2 and M_3 generations

Only SA 1 mM (2h), SA 1 mM (8h), EMS 0.5% (4h) and EMS 1.0% (2h) were recovered after water stress treatment in M_1 and produced seed, screening was carried out in the M_2 and M_3 generations whereby drought stress was only performed in M_3 while M_2 was used to produce seeds for germplasm collection purposes.

Days taken to germinate by M_2 and M_3 were on average four days, which was similar to that of the controls and M_1 plants. The germination rates for the M_2 and M_3 were also recorded as shown in figure 3.3.5A and figure 3.4.5B for SA and EMS treatments respectively. Compared to M_1 there was a significant increase in germination rate in both treatments.

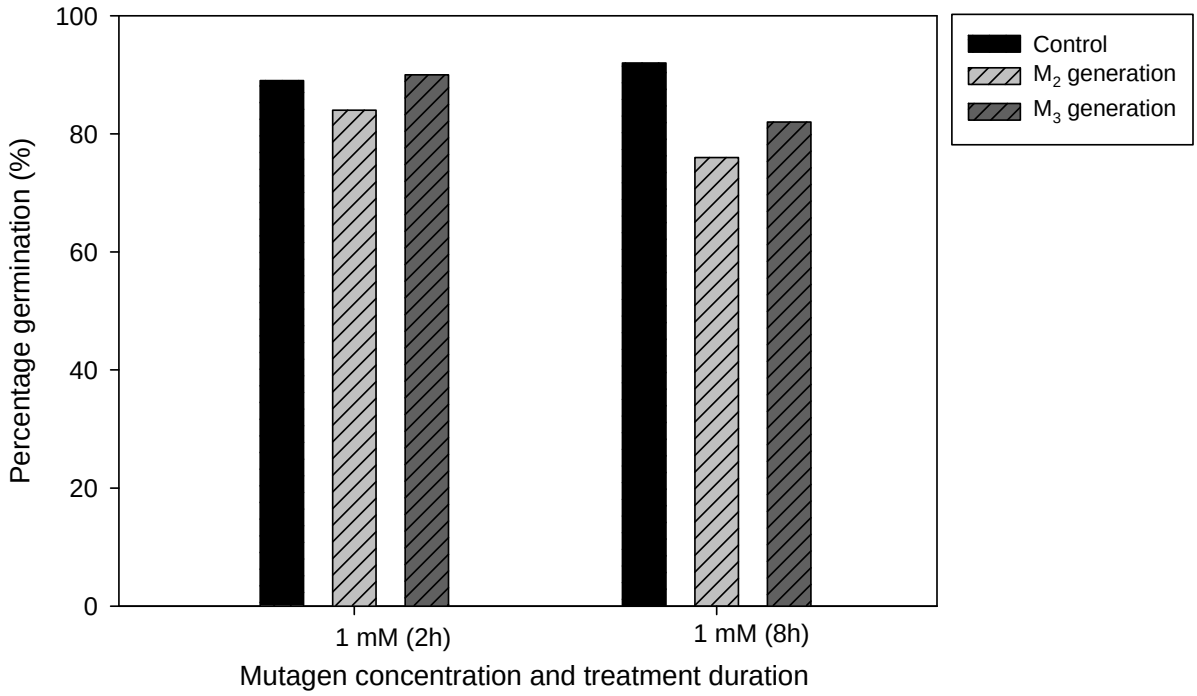

Figure 3.3.5A: Germination rate of SA treated mutant plants (M_2 and M_3) generations.

There was a slight increase in germination rate in the M_3 with 90% and 82% germination rate in SA 1 mM (2h) and SA 1 mM (8h) respectively compared to M_2 generation with 84% and 76% germination rate in SA 1 mM (2h) and SA 1 mM (8h) respectively. In both cases the SA 1 mM (2h) treatment demonstrated a higher germination rate compared to the SA 1 mM (8h) treatment.

Similar increases in germination rate was observed in the EMS treatments, whereby recorded germination rates in the M_3 were 88% and 86% in the EMS 0.5% (4h) and EMS 1% (2h) treatments respectively, when compared with M_2 generation with respective germination rates of 80% and 82% with the SA 1 mM (2h) and SA 1 mM (8h) treatments respectively. Unlike in SA treatments, the EMS treatments showed different germination rates, for example EMS 1% (2h) had a higher germination rate in M_2 and lower germination rate in M_3, while EMS 0.5% (4h) had a lower germination rate in M_2 and higher germination rate in the M_3 generation.

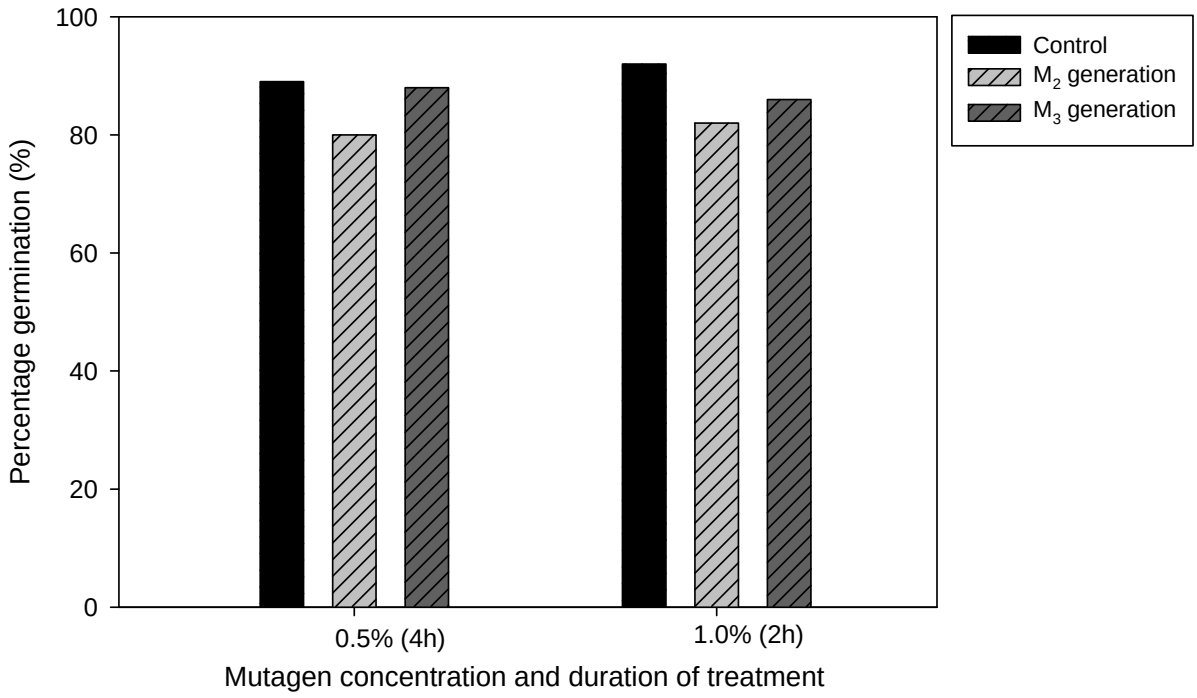

Figure 3.3.5B: Germination rate of EMS treated mutant plants (M_2 and M_3) generations.

To select for mutants that express drought tolerance, M_3 plants were exposed to water stress after 2 to 3 weeks of visually evaluating seedling emergence. This was done by terminating water

supply in all treatments and negative controls, while positive controls received normal watering. Using a modified gravimetric formula from Black (1965) the water moisture content during and after drought stress was monitored and recorded (Table 3.4.5A).

Table 3.3.5A: The average moisture content before and after drought stress across all treatments (M_3).

Mutagen	Water content before stress (ml)	Water content after stress (ml)	Percentage water loss (%)
SA 1 mM (2h)	1.94±0.5	1.09±0.5	44±0.5
SA 1 mM (8h)	1.91±0.5	1.05±0.5	45±0.5
EMS 0.5% (4h)	1.93±0.5	1.03±0.5	47±0.5
EMS 1% (2h)	1.81±0.5	0.91±0.5	50±0.5

The performance of M_2 was monitored in order to produce enough seeds for germplasm collection purposes, while the M_3 plants performance under drought stress was observed and the survival rates calculated (Figure 3.3.5C and Figure 3.3.5D). Plants considered as putative water stress tolerant were scored on a scale of 1-3 for their phenotypic symptoms such as wilting, chlorosis and leaf rolling (Small 2013). Compared to M_1 (Figures 3.3.2B and 3.3.2C) results show an equal increase in survival rate in both SA and EMS treatments regardless of their concentrations. There was an approximately 50% survival rate in both SA and EMS in M_3 drought screening (Figure 3.3.5C and Figure 3.3.5D).

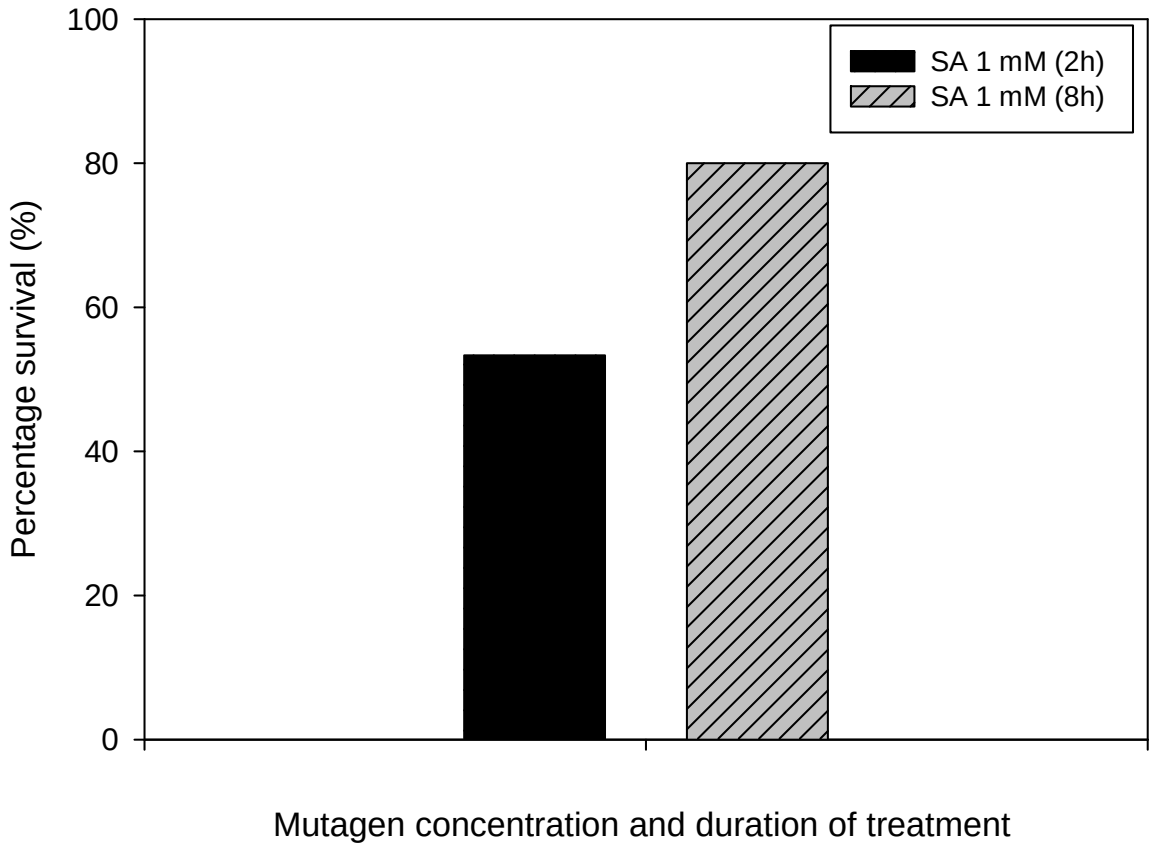

Figure 3.3.5C: SA treatment survival rate (M_3 generation).

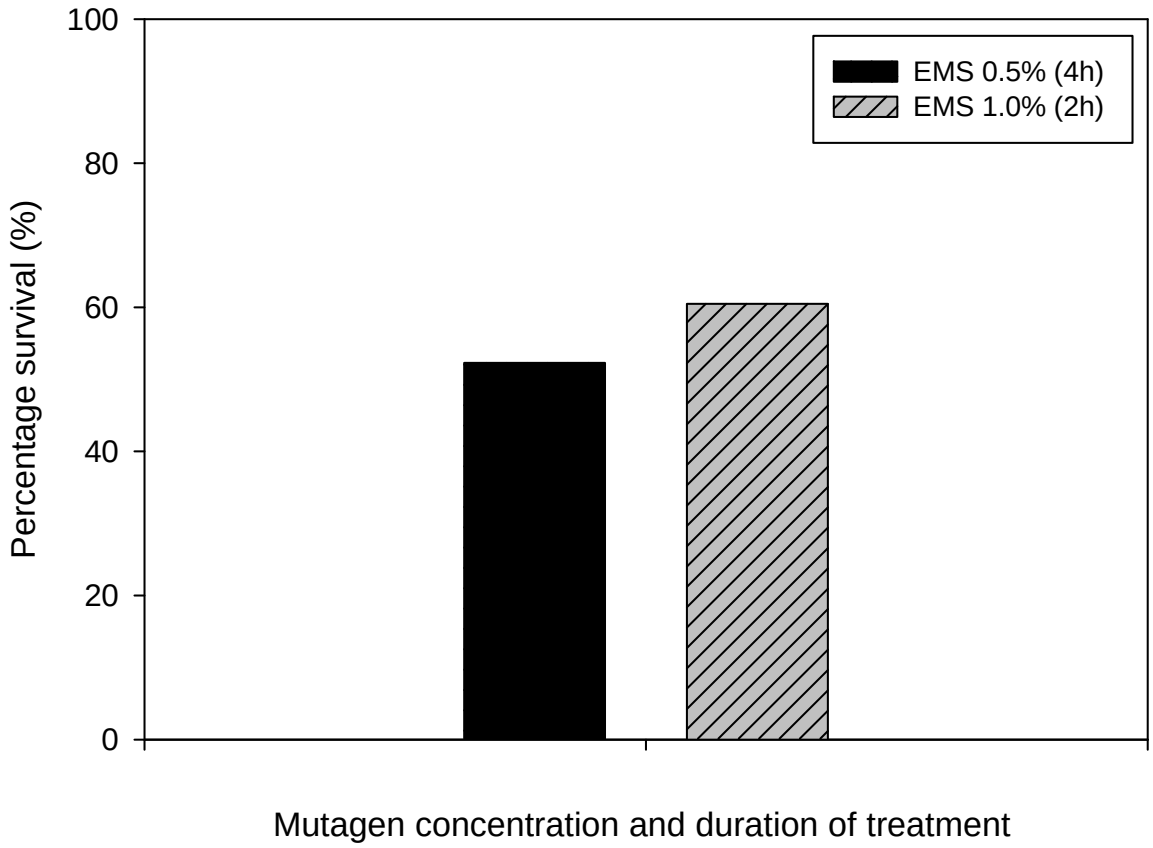

Figure 3.3.5D: EMS treatment survival rate (M_3 generation).

The M_2 and M_3 progenies expressed stunted growth similar to their mutagenic M_1 parents, with an average height of 45 cm compared to the controls (average height of 80cm). Yield related traits showed an increase in seed production for the M_2 and M_3 when compared to the M_1 parent generation. Details of days to flowering, number of seeds and seed weight are presented in Table 3.3.5B and the germplasm database collection is also recorded. Late maturity and partial or total sterility were noticeable in M_3 after drought stress unlike in the M_2 generation where no drought stress was induced. Harvested seeds were recorded and stored for future use.

Table 3.3.5B: Days to flowering, number of seeds produced and average seed weight obtained in the M_2 and M_3 generations.

Accession number	Treatment	Days to flowering		Number of seeds		Thousand Kernel Weight (TKW) (g)	
		M_2	M_3	M_2	M_3	M_2	M_3
SU2012/SA/1/2/16	SA 1 mM (2h) 16	270	270	3710	4502	0.0293	0.0289
SU2012/SA/1/8/21	SA 1 mM (8h) 21	298	290	2156	3089	0.0269	0.0253
SU2012/EMS/0.5/4/7	EMS 0.5% (4h) 7	226	230	2608	3012	0.0301	0.0329
SU2012/EMS/1/2/12	EMS 1% (2h) 12	214	220	2689	3208	0.0272	0.0280
Control	Vernalized	100	100	-	-	0.0323	0.0347

3.4. Discussion and conclusion

3.4.1. The prime strategy in mutation-based breeding

The main strategy in mutation-based breeding is to improve plant varieties by changing major polygenic traits which are sometimes difficult to achieve through conventional plant breeding strategies (Ahloowalia *et al*. 2004). In several mutation-derived plant varieties, the changed traits have resulted in synergistic effects by increasing the quality and yield of the crop, (Ahloowalia *et al*. 2004). Most importantly, induced mutagenesis is considered as an effective way to increase responses in crops against biotic and abiotic stresses. Experiments described in this study demonstrated the use of chemical mutagens to produce putative mutant lines of wheat (*T. aestivum*) with improved water stress tolerance.

3.4.2. Mutagenic effects of sodium azide (SA) and ethylmethanosulfonate (EMS)

SA is a strong mutagen and growth of plant parts are strongly inhibited with increasing its concentration and treatment duration when exposed (Al-Qurainy 2009). The mutational effects of SA has been observed in other plants and it is very effective in inducing mutations with respect to germination percentage, seedling height, seedling survival and yield per plant (Adamu and Aliyu 2007). The potent mutagenicity of SA in wheat, as demonstrated in this study, shows that SA can be an effective, highly specific mutagen for use under controlled conditions for generating potential useful variants in hexaploid wheat. A reduced number of mutants recovered with azide as compared to other mutagens may be compensated for by the specificity of azide and by a lack of the chromosomal aberrations that may accompany the use of other mutagens (Rines 1985). Literature suggests that the reduction in seedling survival is ascribed to physiological disturbances and cytogenetic damage (Sato and Gaul 1967). The greater sensitivity at higher mutagenic concentrations has been attributed to numerous factors such as changes in the metabolic activity of the cells, inhibitory effects of mutagens etc. (Krishna *et al*. 1984). In this study ‘TugelaDN’ seeds were treated with SA and EMS and the immediate effects of mutagenic treatments were measured in terms of biological damage caused in the M_1 generation. SA and EMS treatments brought about the reduction in seed germination, pollen fertility and

survival at maturity. These reductions, with low survival rates were found to be concentration and treatment period dependent.

3.4.3. *N*-Nitroso-*N*-methylurea (NMU) and Maleic Hydrazide (MH)

The results suggest that 'TugelaDN' seeds were not particularly sensitive to NMU and MH mutagens in terms of germination rates, at least with all the concentrations and the treatment durations used in this study. Both mutagens did not reduce the germination percentage much when compared to control. However no plants could be produced after drought treatment. Hence, it may still be possible to increase the concentration and duration of treatment to induce more mutations. Previous studies have reported that the application of MH induces a low frequency of chlorophyll mutations when applied in higher plants. However MH has been claimed to be a weaker mutagen as compared to NMU (Swietlinska and Zuk 1978, Ponnampalam *et al*. 1983). Evidence from studies suggest that NMU is not a viable chemical mutagen for application in wheat, since no resistance could be obtained to the rust *Puccinia graminis* after treatment of wheat plants as a result on MH induction (Swietlinska and Zuk 1978). Nonetheless, there are unanswered and pending questions about the activity of both compounds in terms of clastogenic effects on plant cells (Marcano *et al*. 2004, Cortes *et al*. 1985).

3.4.4. Morphological traits associated with mutagenic wheat lines

Morphological traits were among the earliest markers used in germplasm management, but unlike molecular markers they have a number of limitations such as low heritability, late expression, and susceptibility to environmental influences (Beyene *et al*. 2005). The analysis of different studied traits in this study during drought screening revealed highly significant differences amongst different mutant accessions such as the germination rate, days to flowering, plant height and seed production suggesting that there was a high degree of phenotypic diversity among the obtained mutants.

3.4.4.1. Germination rate

Results in this study showed seed germination of *T. aestivum* at various concentrations of SA and EMS varies, and percentage germination decreased as the concentration of both mutagens increases and also with increased treatment duration. From a germination percentage aspect, mutagens ranked in the following descending order: NMU=MH>EMS>SA. Therefore, SA had the highest lethality concentration in this experiment to an extent that no seeds treated with 10 mM SA germinated. The results of 10 mM SA treatments are probably due to high levels of toxicity as a result of high concentration of SA applied. Hence, to obtain the highest variability and number of suitable mutants, it is inevitable to use lower concentrations of this mutagen over shorter treatment periods. This study suggests the efficiency of chemical mutagens and present SA as the most efficient mutagen followed by EMS. Some mutants were eliminated from the population in the first generation, or they became sterile if they did survive (Table 3.3.4). This is due to mutagenic effects on plant genes and/or chromosomal aberrations as reported in literature (Rines 1985, Gichner *et al*. 2000, Swietlinska and Zuk 1978, Ponnampalam *et al*. 1983, Cortes *et al*. 1985). The extent of reduction in growth is related to the mechanism of action for a given mutagen. Mutagens may inhibit an energy supply system resulting in the inhibition of mitosis which can be associated with seedling growth depression (Afsar *et al*. 1980). Seed's physiological conditions during treatment greatly influence the magnitude of growth depression (Afsar *et al*. 1980). The 0.5% (EMS) for 4h, 1% (EMS) for 2h, 1 mM (SA) for 2h, 1 mM (SA) for 8h, were considered as optimum treatment conditions.

3.4.4.2. Derived mutants with stunted growth

The height of a plant is a key component of plant architecture, as a result of abiotic stresses plants would respond by delaying plant growth and development (Gasperini *et al*. 2012). Results have shown that putative mutant wheat plants demonstrated shorter architecture (Figure 3.3.3A) with approximately half the height of the untreated matured controls. Such findings primarily support the morphological adaptive strategy gained by these plants as the result of successful induction of mutations. Stunted growth can resort as a cost saving mechanism and thus a useful trait for which wheat producers can target drought tolerance in their cultivars (Rana *et al* 2013). Literature suggests short height genotypes as the result of induced mutations in cereals such as

wheat, maize, rice and barley have shown to contribute significantly to increasing grain yield because of their resistance to lodging and high planting density (Ahloowalia *et al*. 2004). The short height trait also has indicated to allow the use of relatively high doses of nitrogen during fertilizer application. These attributes could be developed and used a morphological adaptation of plants under stress. Most importantly, the derived mutant lines have an advantage due to the fact that they were accompanied by seeding (grain formation) as indicated on figure 3.3.3C and table 3.3.4.

3.4.4.3. Late maturity

Mutant plants developed in this study were found to be not only semi-dwarf but also with delayed maturity as they took longer to mature when compared to the control plants (Table 3.3.4). This might be explained by a delayed germination of the mutants as a possible result to additional mutations induced by the mutagen treatment.

In conclusion, the study on mutagen treatments indicated an overall reduced germination rate with increasing mutagen concentration and treatment duration. A successful phenotypic screening allowed the identification of 12 derived mutant lines associated with water stress tolerance. A considerable reduction in plant height was noted in the 7 SA treated and 5 EMS treated derived mutant plants that survived drought stress, with these plants reaching only the height of approximately half that of the controls. Derived mutant wheat lines also showed slower growth rates and delayed maturity with seeds displaying wrinkled morphology and lower average seed mass when compared to controls. Such traits were verified in the M_2 and M_3 generations with the exception of higher germination rate and higher seed production in M_2 and M_3 generations. Drought tolerance conducted on M_3, phenotypically confirmed the findings found in the mutagenesis experiments performed in M_1 generation. The 0.5% (EMS) 4h, 1% (EMS) 2h, 1 mM (SA) 2h and 1 mM (SA) 8h were considered as optimum treatment conditions used in this study.

Drought screening could be synchronized with when crops face drought in South Africa (where study was conducted) and this may not necessarily be at germination when the plants are at their weakest stage. However, these lines may further be evaluated under different growth stages in

order to select the useful ones. Additionally, drought simulation under rainout shelters would also give very interesting data.

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Chapter 4: AFLP profiling of mutagenic wheat lines to assess the extent of induced mutations

4.1. Introduction

The exploitation of genetic variability is very important for the development of new cultivars and improvement of crops (Khan *et al*. 2005, Beyene *et al*. 2005, Jain 2012). Genetic variability can be achieved spontaneously or induced by mutagen treatments. However, the frequency rate of spontaneous mutations is very low and thus unreliable for plant breeding in developing new cultivars. On the other hand, mutation breeding through mutagen treatment has been successful in the past during the development of new mutant cultivars expressing desirable traits (Jain 2012).

Induced mutagenesis can also be beneficial in the development of varieties for use in sustainable agriculture as it is a cost-effective, complementary approach to crop improvement. The number of physical and chemical mutagens used in mutation breeding is numerous and continuously increasing (Hassan *et al*. 2012). Radiation has been the most frequently used method for developing mutant varieties (Alghamdi *et al*. 2010, Tomlekova 2010), while the use of chemical mutagens was relatively uncommon in the past. A large range of both chemical and physical mutagens have been investigated for their use in crop improvement and their proper concentrations has been established (Tomlekova 2010). According to Juchimiuk *et al*. (2007), the ethylated agents such as ethyl methane sulphonate (EMS), also used in this study, have been found to be more effective than physical mutagens in a wide variety of crops including wheat.

Induced mutagenesis can also be employed to improve diversity in crops known to suffer from limited genetic diversity such as bread wheat (*T. aestivum*) (Larik *et al*. 2009, Larik and Hafiz 1981, Khan *et al*. 2005, Roychowdhury and Tah 2011, Emrani *et al*. 2011). Over the years, wheat breeders invested heavily to extend the crops' genetic diversity and analyzing significant traits to accelerate progress. However, the very large size (16,000 MB) and polyploid complexity of the bread wheat genome has always been a substantial barrier to genome analysis (Brenchley *et al*. 2012). Knowledge of genetic relationships between different genotypes is important in order to understand the genetic variability available and its potential application in plant breeding programs (Beyene *et al*. 2005) as it largely determines the future prospects of any breeding program.

The inheritance of critical economic traits such as drought tolerance, quality, yield, adaptation, and disease resistance can be understood through the analysis of a wider range of induced mutations (Emrani *et al*. 2011, Khan *et al*. 2005). Most importantly, any germplasm developed through mutation breeding must essentially be analyzed genotypically. Improvements in marker detection systems and the techniques used to identify markers linked to useful traits, has enabled great advances in recent years (Mueller and Wolfenbarger 1999) with amplified fragment length polymorphism (AFLP) being one amongst most popular marker systems in cereal crops. AFLPs have been used to monitor DNA sequence variation in and among the species after introducing new and favorable traits from related species (Mba and Tohme 2005, Mueller and Wolfenbarger 1999, Legesse *et al*. 2006).

In this study, AFLP was used as the method of choice because it produces large number of bands after the amplification step and no prior knowledge of genome sequence was needed unlike with markers like Restriction Fragment Length Polymorphism (RFLP) and Simple Sequence Repeat (SSR) (Kiula *et al*. 2006, Beyene *et al*. 2005, Park *et al*. 2006, Westengen *et al*. 2005, Dasmahapatra *et al*. 2009, Legesse *et al*. 2006). Even though no prior sequence information is required, the origin of the induced changes is likely to be within the *EcoR*1 and *Mse*I restriction sites upon which the AFLP protocol is based (Vos *et al*. 1995). Therefore, using AFLPs, the aim of this study was to indicate what level of genetic diversity had been introduced by means of chemical induced mutagenesis on a set of derived mutant wheat lines associated with improved levels of drought tolerance.

4.2. Materials and methods

4.2.1. Plant material

The putative drought tolerant mutant lines analyzed in this investigation were obtained through chemical induced mutagenesis using 'TugelaDN' (Tugela/SA1684*7) seeds. Four chemical mutagens namely: Sodium azide (SA); Ethyl methanesulfonate (EMS); Maleic hydrazide (MH); and *N*-methyl-*N*-nitrosourea (MNU), were used in the mutagenesis experiments, while distilled water (dH_2O) was used for the controls. Seeds were firstly pre-soaked in 0.1 M phosphate buffer (pH 7.5) at 4^{o}C for 16h (Olsen *et al*. 1993, Lee *et al*. 2011, Kim *et al*. 2006). Thereafter the buffer was decanted and fresh buffer was added and soaked at room temperature for 8h. After the soaking steps, seeds were treated in oxygenated solutions of SA (0.1 mM, 1.0 mM and 10 mM), EMS (0.1%, 0.5%, 1.0% v/v), NMU (0.5 mM, 1.0 mM, 2.0 mM) and MH (0.5 mM, 1.0 mM, 2.0 mM) with the treatment durations of 2h, 4h and 8h for each concentration. After each treatment, seeds were rinsed to remove excess mutagens (Al-Qurainy 2009) and left to dry (Rines 1985). Seeds were sown into plastic trays containing compost soil mix as described by Lee *et al*. (2011), watered and placed in the green house at Welgevallen experimental farm (Stellenbosch University) during January – September 2012 with natural day/night regimes.

To select for mutants that express water stress tolerance, plants were exposed to water stress after 2 to 3 weeks of visually evaluating seedling emergence (Chapter 3). This was done by terminating water supply in all treatments and negative controls, while positive controls received normal watering. Using a modified gravimetric formula from Black (1965) the water moisture content during and after drought stress was monitored, calculated and recorded (Chapter 3). The performance and phenotypic traits such as germination rate, survival rate, height and yield of the M_1 plants were also constantly monitored and recorded. Plants considered putative drought tolerant were scored on a scale of 1-3 for their phenotypic symptoms such as wilting, chlorosis and leaf rolling (Small 2013). Seven SA and five EMS derived mutants (M_1) were recovered after drought stress and thus used for the AFLP analysis (Table 4.3.1).

4.2.2. Genomic DNA extraction and DNA purification

Leaf tissue was harvested in bulk from the seven SA and five EMS derived mutants (M_1) lines that were recovered after water stress treatment. Pestle and mortars were used to grind plant material in liquid nitrogen. Genomic DNA was extracted from selected M_1 plants using DNAzol® (Invitrogen) following the manufacture`s procedure. DNA quantification was determined using the ND-1000 spectrophotometer (NanoDrop Technologies), and the purity of the DNA sample was calculated using the 260/280 nm and 260/230 nm ratios (Table 4.3.1). Purity and integrity were visually assessed after resolving the samples on a 2% agarose gel (Figure 4.3.1A) with a 1 kb ladder used as a size marker (Fermentas). Gel images were captured with a MULTI-GENIUS Bio-Imaging System.

4.2.3. AFLP analysis

AFLP analysis was performed according to the protocol of Vos *et al*. (1995) with minor modifications. AFLP template preparation was performed using the AFLP® Template Preparation Kit from LI-COR Biosciences according to the manufacturers' instructions. Genomic DNA of the putative drought tolerant mutant wheat lines (approximately 100 ng/µl) was digested with restriction enzymes *EcoR*1 and *Mse*I. In a second step the adaptor sequences were ligated to the restricted DNA fragments using the *EcoR*I and *Mse*I adapter mix. Using the AFLP® Pre-amp primer mix provided in the kit, the pre-amplification cycle profile was as follows; 94ºC for 30 sec, 56ºC for 1 min and 72ºC for 1 min for 20 cycles followed by a soak at 4ºC. For confirmation of pre-amplification, samples were run on 1.5% agarose (w/v) (Figure 4.3.1B).

The selective amplification was performed using the IRDye®Fluorescent AFLP®kit for Large Plant Genome Analysis (LI-COR Biosciences) with a cycle profile as follows; one cycle at 94ºC for 30 sec, 65ºC for 30 sec, 72ºC for 1 min and 94ºC for 30 sec; followed by 12 cycles of successively lowering annealing temperature of 65ºC (reduced by 0.7ºC per cycle), while the denaturing (94ºC) and amplification (72ºC) temperatures remained the same. This was followed by 23 cycles of 94ºC for 30 sec, 56ºC for 30 sec, 72ºC for 1 min and a soak at 4ºC. All

polymerase chain reactions (PCRs) were performed using a GeneAmp® PCR 2700 (Applied Biosystems).

Seven primer combinations were screened using two samples that were expected to represent a high level of genetic diversity due to difference in mutagen concentration and treatment duration. Each *Mse*I and *EcoR*I primer has two extra nucleotide bases added to the 3' end to make them more specific. Each primer was abbreviated using the first letter of either *Mse*I (M) or *EcoR*I (E) and the two extra nucleotides. Using this abbreviation system, the following primer pairs: MTT/ECA; MTT/ECG; MTT/EGC; MTG/EGG; MTG/ECC; MTG/ECT and MTG/EGC were screened (Figure 4.3.2A). The three primer combinations i.e. MTT/ECG, MTG/ECT and MTG/EGC (Table 4.3.2) with the highest polymorphism rates and large numbers of clearly scorable fragments were selected to analyze the full set of 12 mutant lines. Percentage polymorphism was worked out by dividing the number of scored polymorphic bands by the number of constituent bands (Equation 4.2.3).

$$\text{Percentage polymorphism (P \%)} = \frac{\text{number of scored polymorphic bands}}{\text{number of constituent bands}} \qquad \text{(Equation 4.2.3)}$$

An equal volume (10µl) of loading solution (LI-COR) was added to each selective amplification reaction. Before each gel was loaded, the samples, including loading buffer, were denatured at 94°C for 3 min. A volume of 0.8 µl was loaded with an 8-channel syringe (Hamilton, Reno, Nevada) onto 8% Long Ranger polyacrylamide gels (BMA, Rockland, ME, USA). The composition of the polyacrylamide gels included the 20 ml 8% (v/w) Long Ranger polyacrylamide gel solution, 150 µl 10x ammonium persulphate and 15 µl TEMED as previously described by Myburg *et al*. (2001). Electrophoresis and detection of AFLP fragments were performed on the LI-COR NEN® Model 4300 DNA analyzer system in order to separate AFLP profiles. After the gel electrophoresis, AFLP images were saved in 16-bit TIF format for image analysis (Figure 4.3.2B).

4.2.4. AFLP image and data analysis

The resulting AFLP profiles were assessed and scored visually for the presence/absence of AFLP bands and only unambiguously scored bands were used in the analyses. The Saga™ Automated AFLP Analysis Software (LI-COR Biosciences) was also used for analysis of the TIF output files, followed by manual editing to make corrections to the automated score where necessary. The binary scoring system that recorded the presence of bands as 1 and absence of bands as 0 was applied during scoring of AFLP profiles (Appendix A). The matrices of binary data were constructed with rows equal to mutant derived lines, and columns equal to distinct bands. The data matrix was used to perform cluster analysis (dendrogram) and pairwise genetic distances (GDs) of the 12 accessions (mutant lines) and control by using the Phylogenetic Analysis Using Parsimony (PAUP) software program (Swofford 2003). The pairwise line GDs were computed within each set of the AFLP marker system using the formula given by Nei (1972). From these matrices of dissimilarity coefficients, the mean genetic distances was calculated.

4.2.5. AFLP selection and excision of polymorphic bands (Band recovery)

Primer combinations (MTT/ECG, MTG/ECT and MTG/EGC) were selected based on number of bands generated, clarity and amount of information gained, even distribution of bands along the gel with minimal background signal and highest level of polymorphism (Table 4.3.2). The AFLPs were then re-run with selected primer combinations using 0.4 mm polyacrylamide gels (40 ml 8% Long ranger polyacrylamide gel solution, 300 µl 10x ammonium persulphate and 30 µl TEMED) (Myburg *et al*. 2001). The movement of bands was monitored until the 200 bp band marker was visible. The LI-COR machine was then stopped, and the gel was scanned using the Odyssey infrared imaging scanner system (LI-COR® Biosciences, Lincoln, NB). The Odyssey scanner produced an image of the AFLP gel (Figure 4.3.3A), which was then printed in actual size using an A4 page and aligned under the gel. Using a clean scalpel, gel fragments containing previously identified bands of interest were excised and placed individually into PCR tubes containing 20 ml of dH_2O. After the excision, a second scan was taken to confirm absence of excised bands (Figure 4.3.3B). DNA fragments were eluted through freeze-thaw cycles as suggested by the AFLP® Expression Analysis Kit (LI-COR Biosciences, Lincoln, NB). The elute

was used as template for re-amplification using primers corresponding to the original primer combination used in selective AFLP amplification with modified reactions conditions applied in order to obtain PCR products. The quality of the PCR products was verified by separating products on 1.5% (w/v) high melting point agarose gel (SeaKem® LE Agarose). If more than one band was seen on the agarose gel, the band corresponding to the original fragment size was cut from the agarose. PCR fragments sizes were constantly compared to the original fragment sizes on the AFLP gels before ligated into the pTZ57R/T vector (Fermentas, Life sciences) for cloning.

4.2.6. Cloning and sequencing

The re-amplified products were either sequenced directly or first cloned into the pTZ57R/T vector using an InsTAclone™ PCR Cloning Kit (Fermentas). Using a MinElute® PCR Purification Kit (Qiagen) samples were firstly cleaned before cloning in order to enhance cloning efficiency. The ligation mixes were prepared using; T4 DNA ligase (1 µl), 10x T4 DNA ligase buffer (1 µl), deionized sterile H_2O (5.5 µl), purified cloning vector (pTZ57R/T) (1 µl) and an insert PCR product (1.5 µl). The DH5α *E.coli* strain was used to prepare competent cells (Inoue *et al*. 1990). Standard cloning procedures were followed using the Fermentas cloning protocol (Thermo Scientific). Plates containing ampicillin (100 µg/ml) were prepared and equilibrated at room temperature before use. The ligation reactions were gently mixed with the competent cells, placed in ice for 20 mins and heat shocked for 50 sec. SOC medium (1g Tryptone, 0.25g Yeast extract, 1 M NaCl, 1 M KCL) was then added to the ligation mixes and incubated at 37ºC for 90 mins. IPTG (0.1 M) and X-gal (20 mg/ml) were added and spread on the plates on which the transformed ligation mixes were plated and incubated overnight at 37ºC to allow blue and white colonies to be formed. Using the blue-white screening technique, the inserts were confirmed using colony PCR after picking white colonies (putative transformants) from the plates (Gussow and Clackson 1989). The colonies were then allowed to grow overnight in LB (Luria-Bertain) medium (per liter: 10 g Tryptone, 5 g Yeast extract, 8.56 mM NaCl) with 100 µg/ml Ampicillin in an incubation room at 37ºC. The selected colony cultures were also frozen away as 15% (v/v) glycerol stocks for future use.

Colony PCR was performed using 0.2 µM M13 Forward (5`-TGTAAAACGACGGCCAGT-3`) and M13 Reverse (5`-AGGAAACAGCTATGACC-3`) primers or with primers corresponding to the original primer combination used during selective amplification (i.e. MTT/ECG, MTG/ECT and MTG/EGC). The PCR reaction contained the following; 2.5 mM $MgCl_2$, 10x PCR buffer, 100 µM of each dNTP and 1U Taq polymerase. Thermocycling parameters had an initial denaturation step of 94ºC for 2 mins, followed by 25 cycles with denaturation (94ºC), annealing (55ºC) and the extension (72ºC) for 15 sec, 20 sec and 30 sec respectively, final extension at 72ºC for 5 mins and 21ºC on hold. Prior to sequencing, the concentration and integrity of the samples were verified spectrophometrically and visually using a 2% (w/v) agarose gel (Figure 3.3.4) with lambda DNA as concentration standard. One of each clone or direct PCR products were sent to Macrogen Incorporated (Seoul, Korea) for Sanger sequencing using the dye terminator kit.

4.2.7. Sequence analysis and assigning putative identities

Sequences obtained were manually edited using the Geneious® 6.1.6 software (available from http://www.geneious.com/). To circumvent inconsistences and discrepancies due to primer and vector contamination, the first and last 40 bp were removed from each sequence. In order to attain their putative sequence identities, edited sequences were subjected to the BLAST (Basic local Alignment Search Tool algorithms) search function on the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov) (Altschul *et al*. 1990, Altschul *et al*. 1997, Zhang and Madden 1997). Expectation values (E-value) of 10^{-5} and lower were considered significant and hence used (Altschul 1998).

4.3. Results

4.3.1. DNA extraction and quality

After the extraction of genomic DNA, the concentration and integrity of the samples were verified (Table 4.3.1) and visually through separation using a 2% agarose gel (Figure 4.3.1A). The highest DNA concentration was obtained from SA 1 mM (2h) 6 with the concentration of 358.35 ng/µl while the lowest concentration was obtained from SA 1 mM (2h) 16 with the concentration of 43.47 ng/µl.

Sample quality was assessed using the 260/280 nm absorbance ratio. Most DNA samples were of good quality as measurements obtained were close to or higher than 1.8 as the ratio between 1.8 and 2.0 is generally indicative of good quality genomic DNA whereby the ratio below that may indicate the presence of contaminants such as protein, phenol, etc. (NanoDrop, Wilmington, DE).

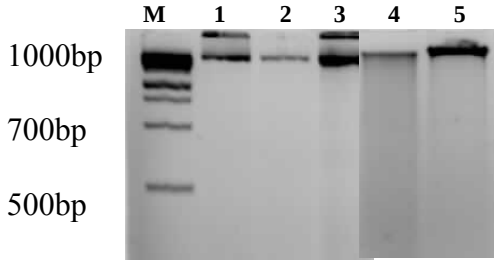

Figure 4.3.1A: Typical quality of purified total genomic DNA extracted as revealed by 2% agarose gel electrophoresis, where **1** = SA 1 mM (2h) 10**; 2** = SA 1 mM (2h) 16**; 3** = SA 1 mM (2h) 6**; 4** = SA 1 mM (2h) 21**; 5** = SA 1 mM (8h) 21; while **M** = 1 kb O'GeneRuler DNA ladder (Thermo Scientific).

Table 4.3.1: Total genomic DNA concentrations (ng/µl) and purity using the 260/280 ratio.

		Total DNA	
Accession number*	**Treatment**	**ng/µl**	**260/280 nm**
SU/2012/SA/1/2/10	SA 1 mM (2h) 10	156.38	2.74
SU/2012/SA/1/2/16	SA 1 mM (2h) 16	43.47	1.72
SU/2012/SA/1/2/6	SA 1 mM (2h) 6	358.35	2.16
SU/2012/SA/1/2/21	SA 1 mM (2h) 21	73.87	1.79
SU/2012/SA/1/8/21	SA 1 mM (8h) 21	212.65	1.26
SU/2012/SA/1/8/1	SA 1 mM (8h) 1	124.59	1.92
SU/2012/SA/1/8/16	SA 1 mM (8h) 16	55.48	2.15
SU/2012/EMS/0.5/4/7	EMS 0.5% (4h) 7	376.35	1.7
SU/2012/EMS/0.5/8/6	EMS 0.5% (8h) 6	96.94	1.7
SU/2012/EMS/0.5/8/11	EMS 0.5% (8h) 11	141.91	1.98
SU/2012/EMS/1/2/12	EMS 1% (2h) 12	58.87	1.07
SU/2012/EMS/1/8/1	EMS 1% (8h) 1	92.92	1.66
Control	nt	124.59	1.55

nt = not treated

* Accessions are denoted as follows: Institution identity/year when experiments were conducted/chemical mutagen applied/mutagen concentration/treatment duration/plant number.

Total genomic DNA purified from all samples was of sufficient quantity (Mean yield = 147.4131 ng/µl) and quality, with little visible contamination. Pre-amplification results for DNA-AFLP analysis (Figure 4.3.1B) yielded fragments with varying sizes.

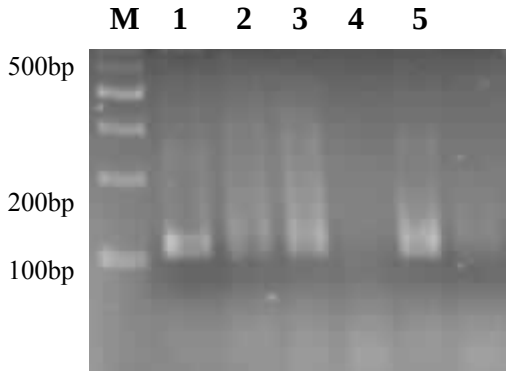

Figure 4.3.1B: Diluted pre-amplification products run on a 1.5% agarose gel during AFLP analysis. **1** = SA 1 mM (2h) 10; **2** = SA 1 mM (2h) 16; **3** = SA 1 mM (2h) 6; **4** = SA 1 mM (2h) 21; **5** = SA 1 mM (8h) 21.

4.3.2. AFLP primer screening and AFLP data analysis.

After the selective amplification were completed, the samples were run on a polyacrylamide gel using the LI-COR gel system in order to screen for the levels of polymorphism. Seven primer combinations were run at a time, with each sample loaded twice to ensure consistency and to remove all ambigous bands (Figure 4.3.2A). Thereafter, three primers (MTG/ECT, MTG/EGC and MTT/EGC) with the highest polymorphism rates and large numbers of clearly scorable fragments were selected to analyze the full set of 12 mutant lines. Several bands were identified by DNA-AFLP analysis with approximately 398 fragments generated from the three different primer combinations that were selected (Table 4.3.2). Of the three primer sets screened, primer pair MTG/ECT showed the highest level of polymorphism (69.5%), while primer pair MTG/EGC gave the least polymorphic fragments (20.6%).

Bands that were selected for further analysis were polymorphic between treated samples or time points and exhibited visually discernible differences (Figure 4.3.2A). Bands of interest were chosen with characterstics such as presence or absence (polymorphic) in comparison to control.

Table 4.3.2: Number of monomorphic and polymorphic fragments obtained after AFLP analysis.

*Mse*I primer (M)	*EcoR*I primer (E)	Monomorphic Fragments	Polymorphic Fragments	Total Fragments	Percentage Polymorphism
MTT	EGC	102	47	149	31.5
MTT	ECA	29	3	32	9.4
MTT	EGC	46	5	51	9.8
MTG	EGC	104	27	131	20.6
MTG	ECT	36	82	118	69.5
MTG	EGG	85	4	89	4.5
MTG	ECC	91	1	92	1.2

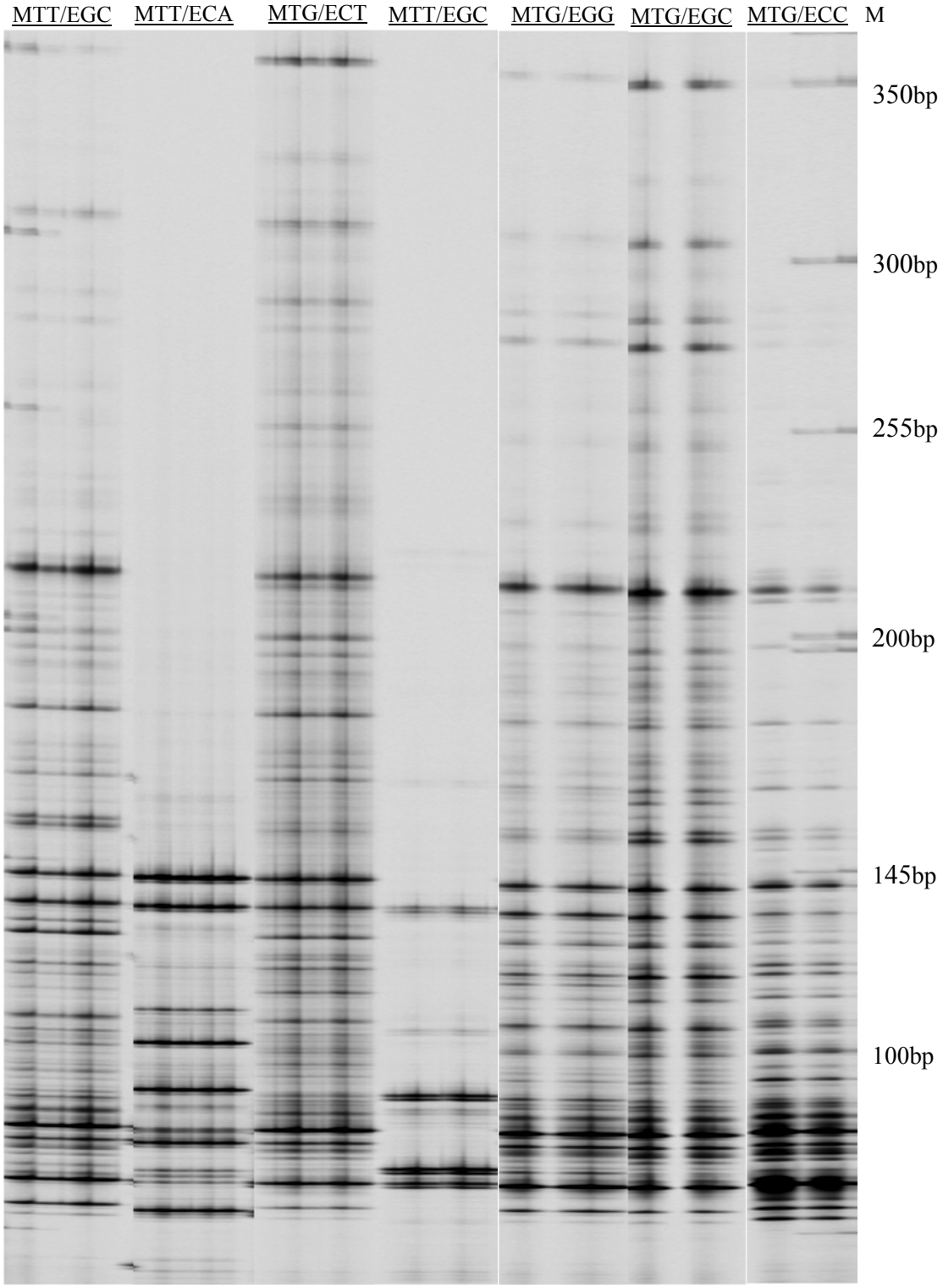

Figure 4.3.2A: AFLP selective amplification with different primer combinations was used during primer screening. IRDye700-labelled was used, with two different samples loaded per primer combination.

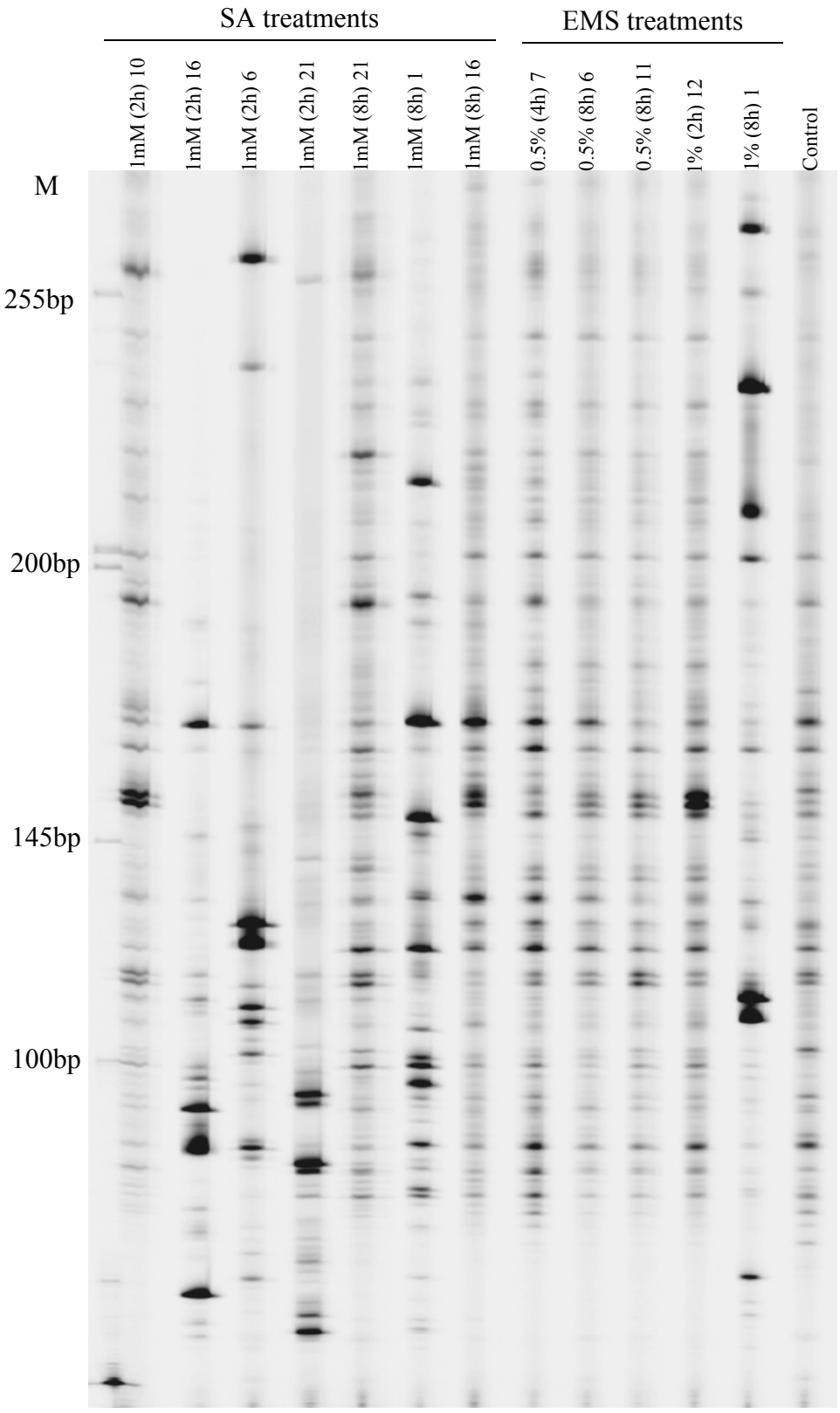

Figure 4.3.2B: Section of a typical AFLP profile obtained from the 13 putative drought tolerant mutants using the MTG/EGC primer combination, **M** = IRDye700-labelled molecular size standard.

4.3.3. Band recovery and identification

The chosen primer combinations (MTT/EGC, MTG/ECT, MTG/EGC) were then re-run on a 0.4 mm gel and bands were excised (Figure 4.3.3).

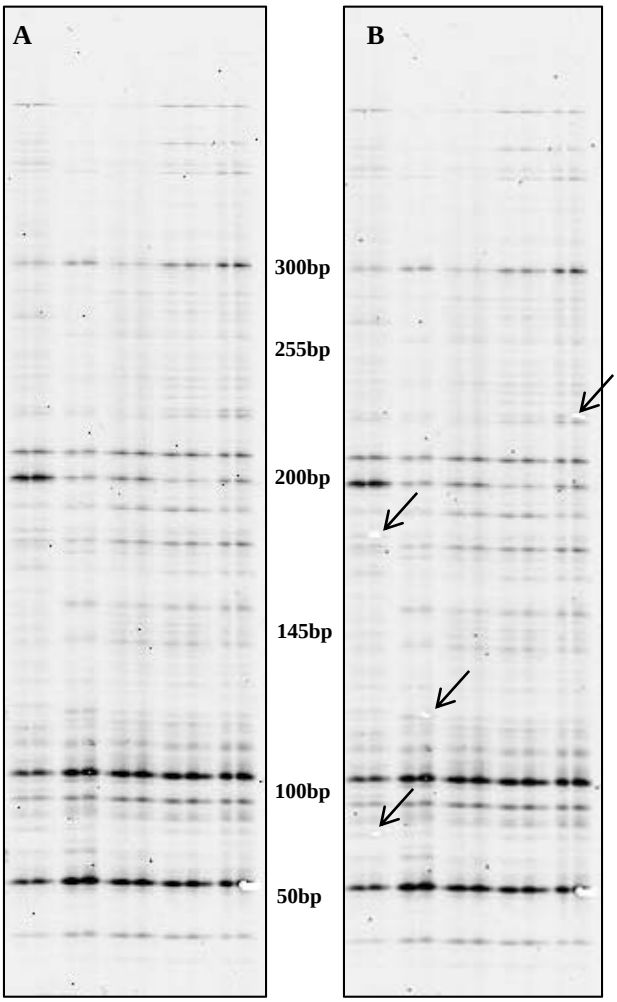

Figure 4.3.3: (A) AFLP gel image produced by the Odyssey scanner; (B) Gel scanned after succesful band excision using MTG/EGC primer combination. Arrows point out a few of the bands that were excised.

4.3.4. Re-amplification of excised bands

The exicised bands were re-amplified (Figure 4.3.4A) to produce PCR products. The PCR products were then cloned and verified through colony PCR from the putative transformants (Figure 4.3.4B) and sequenced in order to assign putative identities.

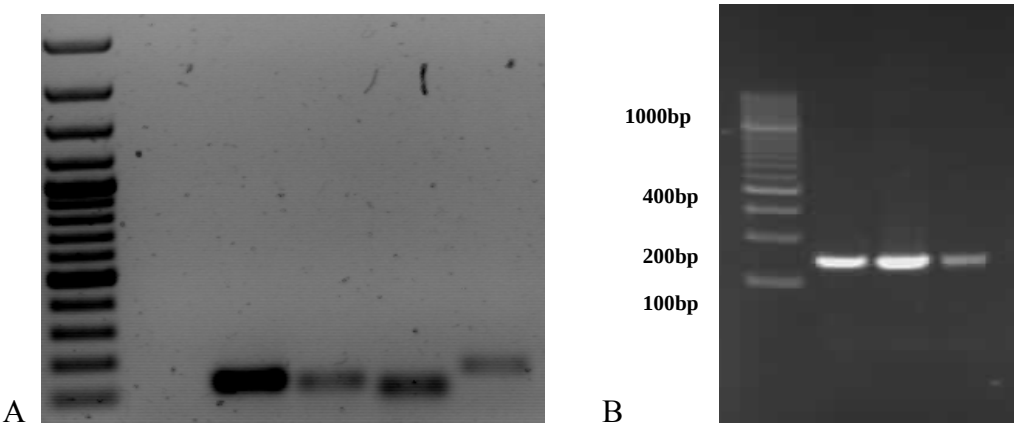

Figure 4.3.4: (A) Re-amplified bands from AFLP gel were run on a 2% agarose gel. Lane 1 contains 100 bp ladder (O'GeneRuler™ Fermentas); (B) Colony PCR products run on a 1.5% agarose gel with band sizes indicated to the left in number of base pairs.

The sizes of the re-amplification products varied and were not always corresponding to the actual size of the products run on polyacrylamide gels due to co-migration of bands (Myburg *et al*. 2001).

4.3.5. Sequencing

After re-amplification and cloning, sequences were obtained and putative identities were assigned to the clones using BLASTn based on sequence similarities. Of the 156 polmorphic bands excised from the gels, 25 were cloned and sequenced for analysis. Of the 25 cloned sequences, one revealed a putative identity to a *Triticum aestivum* isolate AAC/CTG7 scab resistance-linked AFLP fragment gene sequence (Sequence ID: gb|AF354657.1| Length: 143), while two clones identified from the NCBI database had no significant similarity to any characterized gene/sequence in the GenBank. The rest of the sequences (21 clones) revealved no sigificant similarity to any sequence found in GenBank (Table 4.4.5).

Table 4.3.5: Putative identity assigned to submitted clones with respective accession numbers after BLASTn analysis. The length of the fragment and the E-value obtained after BLASTn analysis are indicated.

Clone ID	Sequence identity	Accession	Length (bp)	E-value
MTG/EGC44	*Triticum aestivum* isolate AAC/CTG7 scab resistance-linked AFLP fragment gene sequence	AF354657.1	143	8e-13
MTG/EGC47	No similarity		60	
MTG/EGC48	No similarity		78	
MTG/EGC41	No similarity		85	
MTG/EGC49	No similarity			
MTG/EGC81	No similarity		195	
MTG/EGC38	*Triticum aestivum* chromosome 3B-specific BAC library, contig ctg0382b	FN564429.1	166	1e-24
MTT/ECG35	No similarity		146	
MTT/EGC4	No similarity		112	
MTT/EGC12	No similarity		104	
MTT/EGC31	*Triticum aestivum* clone 3175O8 genomic sequence	JF758496.1	174	5e-05
MTT/EGC111	No similarity		105	
MTT/EGC49	No similarity		136	

Clone ID	Sequence identity	Accession	Length (bp)	E-value
MTT/EGC38	No similarity		489	
MTT/EGC121	No similarity		458	
MTT/EGC15	No similarity		337	
MTT/EGC125	No similarity		240	
MTT/EGC51	No similarity		105	
MTT/EGC55	No similarity		163	
MTT/EGC41	No similarity		105	
MTT/EGC27	No similarity		525	
MTT/EGC28	No similarity		181	
MTT/EGC75	No similarity		193	
MTT/EGC4	No similarity		191	

4.3.6. Genetic distances (GD) and cluster analysis of derived mutants lines (M_1).

In order to assess the number of *EcoR*1 and *Mse*I restriction site changes that were induced through the chemical mutagen treatments, a binary data set was compiled from the obtained AFLP data and analyzed using Nei's minimum genetic distance formulae (Nei 1972) (Appendix A). After the analysis of the binary data set using PAUP program, it was revealed that SA 1 mM (2h) 16 had the highest number of induced total character differences (109) relative to the control of all the SA and EMS derived mutants (Table 4.3.6). Of the EMS treatments, EMS 1% (8h) 1 had the highest number of induced total character differences (87) relative to the control. While the mutant derived wheat lines with the least number of induced changes relative to the control being SA 1 mM (2h) 10, EMS 0.5% (8h) 11 and EMS 1% (2h) 12 (total number of induced changes of 45, 44 and 44 respectively).

Pairwise GD estimates for the 12 mutant lines ranged from a minimum of 0.02817 to a maximum of 0.55869 (Table 4.3.6). The dissimilarity tree produced in this study is presented in Figure 4.3.6. This tree reveals three main groups of mutant lines derived from different treatments. Group I comprising of only SA 1 mM (2h) 10 is clustered distantly from the rest of the mutants lines. Group II consists of five lines with four lines in this group derived from SA induced mutagenesis treatment i.e. SA 1 mM (2h) 16, SA 1 mM (8h) 1, SA 1 mM (2h) 21 and SA 1 mM (2h) 6 and only one EMS treatment i.e. EMS 1% (8h) 1. Group III was the largest one with six derived mutant lines. In this group, four lines originated from the EMS treatments including EMS 0.5% (4h) 7, EMS 0.5% (8h) 6, EMS 0.5% (8h) 11 and EMS 1% (2h) 12, with two from SA derived treatments i.e. SA 1 mM (8h) 21 and SA 1 mM (8h) 16. Of the three groups, group III has the closest association with the control used during the chemical induced mutagenesis experiments.

Table 4.3.6: Pairwise genetic distances (GD) and number of characters using three AFLP primer pair combinations on 12 mutant derived wheat lines and control calculated by PAUP program. (Below diagonal: total character differences; above diagonal: mean character differences).

		1	2	3	4	5	6	7	8	9	10	11	12	13
1	SA 1 mM (2h) 10	-	0.47887	0.25352	0.39906	0.29577	0.44131	0.3615	0.2723	0.2723	0.19249	0.20188	0.40376	0.21127
2	SA 1 mM (2h) 16	102	-	0.38498	0.31455	0.49296	0.2723	0.52113	0.52582	0.49765	0.5493	0.5493	0.23474	0.51174
3	SA 1 mM (2h) 6	54	82	-	0.29577	0.35211	0.34742	0.39906	0.38498	0.31925	0.39906	0.40845	0.30986	0.31455
4	SA 1 mM (2h) 21	85	67	63	-	0.49765	0.26761	0.53521	0.47418	0.38967	0.4507	0.46009	0.21127	0.37559
5	SA 1 mM (8h) 21	63	105	75	106	-	0.46479	0.30047	0.277	0.33333	0.30986	0.30986	0.49296	0.29108
6	SA 1 mM (8h) 1	94	58	74	57	99	-	0.48357	0.52582	0.41315	0.5493	0.5493	0.29108	0.47418
7	SA 1 mM (8h) 16	77	111	85	114	64	103	-	0.23944	0.30516	0.33803	0.33803	0.55869	0.33803
8	EMS 0.5% (4h) 7	58	112	82	101	59	112	51	-	0.24413	0.17371	0.16432	0.46009	0.24883
9	EMS 0.5% (8h) 6	58	106	68	83	71	88	65	52	-	0.21127	0.21127	0.46948	0.33333
10	EMS 0.5% (8h) 11	41	117	85	96	66	117	72	37	45	-	0.02817	0.50235	0.20657
11	EMS 1% (2h) 12	43	117	87	98	66	117	72	35	45	6	-	0.48357	0.18779
12	EMS 1% (8h) 1	86	50	66	45	105	62	119	98	100	107	103	-	0.40845
13	Control	45	109	67	80	62	101	72	53	71	44	40	87	-

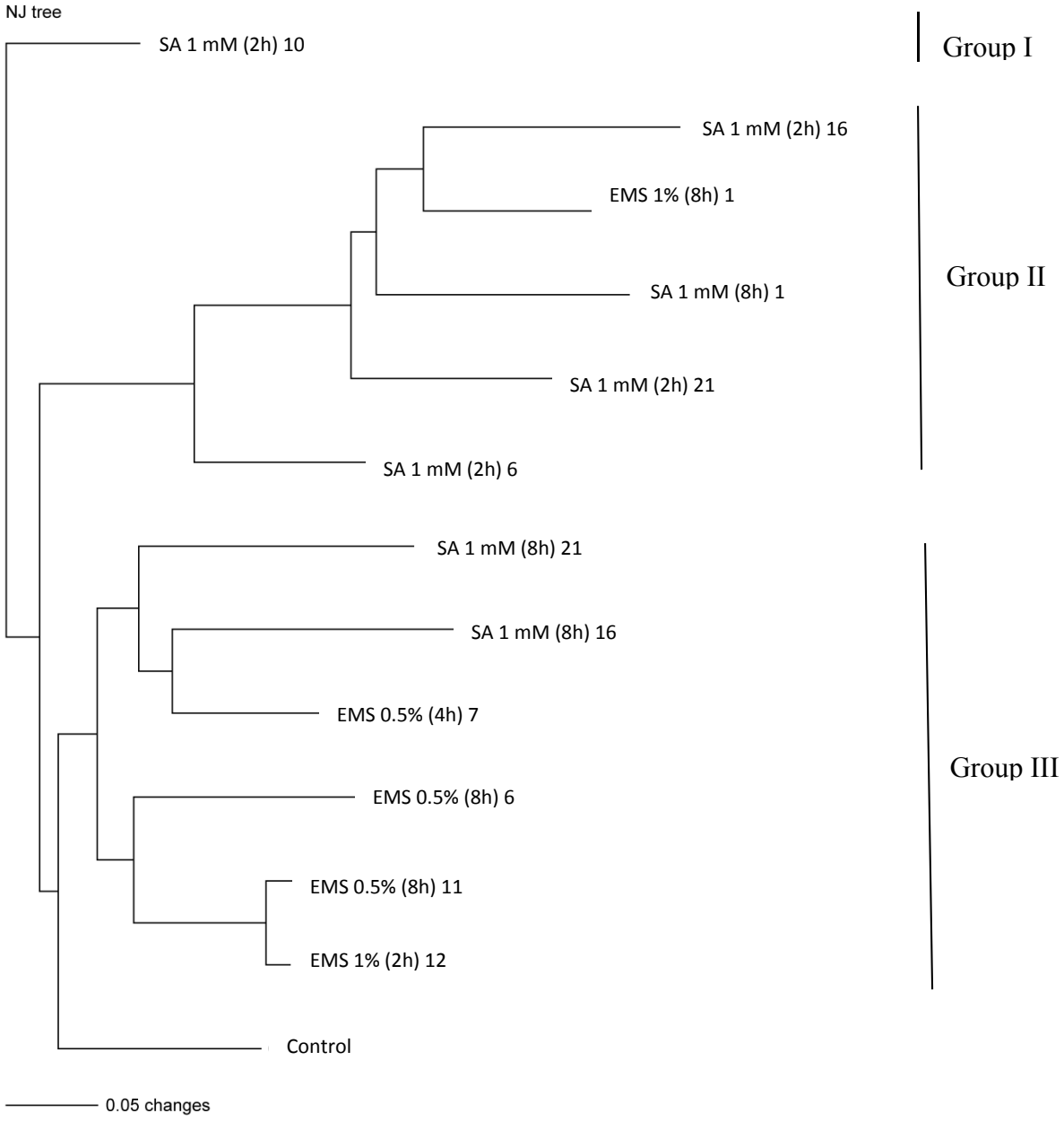

Figure 4.3.6: Neighbor-Joining (NJ) tree based on the AFLP analysis of the 12 mutant derived wheat lines.

4.4. Discussion and conclusion

In this study, we used AFLP markers to characterize a set of 12 mutant derived wheat lines associated with water stress tolerance. A total of 150 AFLP fragments were excised, of these, 25 were cloned and sequenced, and only 3 revealed similarities to sequences in NCBI. All the other fragments were unknown or had no similarities to any previously deposited NCBI sequence (Table 4.3.5). One of the fragments had similarity to a *Triticum aestivum* isolate AAC/CTG7 scab resistance-linked AFLP fragment gene sequence (Sequence ID: gb|AF354657.1| Length: 143), an important disease of wheat.

Fusarium head blight (FHB), also known as Fusarium ear blight (FEB) or scab is a common devastating fungal disease caused by Fusarium *graminearum* that infect members of the grass family Poaceae, including wheat and barley (Buerstmayr *et al*. 2009, Gautam and Macky 2011, Bai and Shaner 2004, Anderson *et al*. 2007, Van Ginkel *et al*. 1996, Anderson *et al*. 2001). In wheat, *Fusarium* infection results in weak dough properties and unsatisfactory baking quality (Gautam and Macky 2011). This infection and colonization of wheat by *F. graminearum* is most prevalent during extended periods of moisture and warm climatic conditions around flowering (Van Ginkel *et al*. 1996, Gautam and Macky 2011). FHB causes the contamination of the crop with toxic fungal secondary metabolites known as deoxynivalenol (DON) which is harmful to animals (Van Egmond 2004, Buerstmayr *et al*. 2009, Van Ginkel *et al*. 1996) by obstructing protein synthesis. There are two components of physiological resistance to FHB: Type I resistance, which is defined as resistance to initial infection by the pathogen, and the second, type II is defined as resistance against the spread of infection within the spike (Schroeder and Christensen 1963). To date, sources of resistance conferring complete resistance to FHB has not been identified in wheat, even though markers linked to the trait are available (Anderson *et al*. 2001, 2007). Unfortunately, due to time constraints, the FHB resistance or the association of the obtained AFLP sequence couldn't be explored further.

The AFLP profiles were also applied to study the association and genetic diversity amongst the derived mutants (Figure 4.3.2B and Table 4.3.6). In the present study, it was found that SA 1 mM (2h) 16 had the highest number of induced total character differences (109) relative to the control of all the SA and EMS derived mutants (Table 4.3.6), while EMS 1% (8h) 1 had the

highest number of induced total character differences (87) of the EMS treatments. The obtained data therefor suggests that a treatment with SA induced more mutations than the EMS treatment (Figure 4.3.6; Table 4.3.6). SA was found to be the most effective mutagen in barley, but didn't increase mutation rate in *Arabidopsis* (Olsen *et al*. 1993). Findings in the present study supported this data, as the SA treatment resulted in the production of fertile plants and induced more changes than any of the tested mutagens. Sarma *et al*. (1979) reported that mutation frequency increase with SA treatment duration, suggesting that more mutations are induced with longer pre-soaking. The current study didn't support this finding as the treatments with the highest number of induced character changes were obtained after a presoaking treatment of only 2h (i.e., SA 1 mM (2h) 16).

Seed treatments with EMS have shown that the frequencies of mutations are dependent on the location of the genes in the genome and the treatment conditions during mutagenesis. Previous studies suggested the mutation frequency increases proportionally with increasing EMS concentration (Hsie *et al*. 1975) and longer exposure times (Alcantara *et al*. 1995). These findings couldn't be corroborated in the present study, since findings were random and no significant correlation between the number of induced changes, EMS treatment concentration or duration of treatment could be demonstrated (Table 4.3.6 and Figure 4.3.6).

In conclusion, the AFLP fragments were very useful to reveal polymorphisms among the different mutagenic wheat lines, thus allow distinguishing between the mutant derived wheat lines, their respective induced changes and distinct genetic profiles. These derived mutants wheat lines can be used for field trials in a natural environment in order to test their capacity when exposed to water deficit conditions (drought). The products from each successive generation can be further fingerprinted and screened using applications such as TILLING (Targeting Induced Local Lesions in Genomes) in order to detect mutations that were induced in specific genes.

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Chapter: 5 Summary

With global warming and climate change being a global concern, innovative adaptive strategies for plant breeders and farmers are required (Barnabas *et al*. 2008, Ahuja *et al*. 2010). Mutation-based breeding is such a strategy that is efficient with low-cost for plant breeders to adapt to the challenges posed by climate change. Especially, chemically induced mutagenesis showed promise in improving drought tolerance using different recommended mutagens at optimum concentrations and treatment durations (Ahloowalia *et al*. 2004, Hassan *et al*. 2012, Jain 2012).

Therefore, the aim of the study was to improve drought tolerance in wheat by means of chemical induced mutagenesis. Experiments detailed in Chapter 3, show the application of chemical mutagens to produce mutant wheat lines with improved drought tolerance. The study compared the mutagenic properties of four chemicals, namely Sodium azide (SA), Ethyl methanesulfonate (EMS), Maleic hydrazide (MH), and *N*-methyl-*N*-nitrosourea (MNU) at different concentrations and treatment durations. The applied mutagen concentrations were as follows: SA (0.1 mM, 1 mM and 10 mM), EMS (0.1%, 0.5%, 1.0% v/v), NMU (0.5 mM, 1.0 mM, 2.0 mM) and MH (1 mM, 2 mM and 4 mM) with treatment durations of 2h, 4h and 8h for each concentration. To select for mutants that express drought tolerance, the M_1 plants were exposed to water stress. NMU and MH treated M_1 plants demonstrated zero survival rates, while a few of the plants treated with SA and EMS survived. In the study, treatments with 0.5% (EMS) 4h, 1% (EMS) 2h, 1 mM (SA) 2h and 1 mM (SA) 8h were considered optimum, since these treatments resulted in fertile plants. However, the chemically derived mutant wheat lines displayed a lower germination rate, delayed maturity, stunted growth and lower than average seed mass when compared to the control. The latter traits were also verified in the M_2 and M_3 generations. The M_2 and M_3 generations also expressed stunted growth and delayed maturity phenotype, but had higher germination rates and produced more seed. Drought tolerance conducted on the M_3 plants confirmed the tolerant phenotype found in the M_1 generation plants.

In Chapter 4, amplified fragment length polymorphism (AFLP) was used, as my method of choice to assess the extent of induced mutations, because of the large number of obtained AFLP fragments (Vos *et al*. 1995). After screening seven primer combinations, three primer combinations (MTT/ECG, MTG/ECT and MTG/EGC) were selected based on number of bands generated, clarity and amount of information gained, even distribution of bands along the gel with minimal background signal and highest level of polymorphism. Using these primer

combinations, AFLP profiling was conducted, where after selected polymorphic bands excised and sequenced characterised. Even though a total of 150 AFLP fragments were excised, only 25 were cloned and sequenced. Of the sequenced clones, only one revealed similarity to a *Triticum aestivum* isolate AAC/CTG7 scab resistance-linked AFLP fragment gene sequence (Sequence ID: gb|AF354657.1| Length: 143). Scab is an important disease of wheat (Anderson *et al*. 2001, Anderson *et al*. 2007, Bai and Shaner 2004, Buerstmayr *et al*. 2009, Gautam and Macky 2011, Van Ginkel *et al*. 1996), but due to time constrains this finding was not investigated further.

The number of chemically induced mutations was also quantified. Using Nei's (1972) minimum pairwise genetic distance, a binary data matrix was constructed and cluster analysis performed. From the binary data matrices analysed using PAUP program, it was revealed that SA 1 mM (2h) 16 had the highest number of induced total character differences (109) relative to the control of all the SA and EMS derived mutants, suggesting that a treatment with 1 mM SA for 2h induced more mutations than any other SA or any of the EMS treatments.

In conclusion, this study succeeded in producing twelve derived mutant wheat lines from 'TugelaDN' with improved drought tolerance using chemical induced mutagenesis. The chemically induced mutagenic lines were phenotypically and genotypically analysed. Even though the chemically derived mutant wheat lines displayed a lower germination rate, delayed maturity, stunted growth and lower average seed mass, they could on average water loss tolerate more drought conditions when compared to the control. AFLP profiling was used to analyse these lines and found that the technology was very useful to reveal polymorphisms among the different mutagenic wheat lines, allowing me to distinguish between the mutant derived wheat lines, their respective induced changes and distinct genetic profiles.

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APPENDIX A

Nei's minimum genetic distance (1972)

SA 1 mM (2h) 10

00000110100111111111111111110111011110100011111111110000000000001000000100000001111111111111111111101111111101000111100000000000000000010001000001001101001111111111111111111011101110001111111111111000001101001000000

SA 1 mM (2h) 16

11000000001110000110000000000111001010000100000000000000000000000000000000000111001010000100000011000000001110000110000000000111001010000100000000000000001111101110111100000111001010111000011000000000000000001111000

SA 1 mM (2h) 6

00100111100111000110010011111001000110000000000000000000000000000000000000000001111111111111111111101111111101000111100000000000000000000000000000000000111100100000111001011110010001100011100011001000000111100000000100

SA 1 mM (2h) 21

00100111101111001110000000000100000000000000000000000000000000000000000000010010011110111100111000000000010000000000000000000000000100001000110001011001001111011111111001001111100111000000000001111000000010000

SA 1 mM (8h) 21

00000100100111111111111111110101011110100011101111110000101011001000000000000100100111111111111111101010111101000111111111000010101100110000000000010010010000001000001100000011111111111110001010010010000000000

SA 1 mM (8h) 1

00100000001111100000100000000011001000000000100000000000000000000000000000100100000001111010101000000000000000000011111000001000000000000000000100

SA 1 mM (8h) 16

000000111001111111111111111111011110111111101111110000101011001000000000000011
100111111111111111111101101011111101111110000101011001000000000000001110010000
001100000000000000000011111111111110001001110010000000000

EMS 0.5% (4h) 7

000001111011111111111111111110011111011111111111110000010100100100000000000011
110111111111111111111100010110111111111111110000010100110100000000000001111011111
111101100011111111100011111111111111000101111001000000000

EMS 0.5% (8h) 6

001001111011111111111011111111111111010000000000000000000000000000000000011
110111111111111111111111111111011010111111111001010101100100011010010011110111
111001100000001000000000111111111111110101011110010000000

EMS 0.5% (8h) 11

000001111011111111111111111011111111011111111111111100101010110010001001000011
110111111111111111111011111111001111111111111100101010110010001001001001111011111
111111111111101111110001111111111111101010111100100000000

EMS 1% (2h) 12

000001111011111111111111111011111111011111111111111100101010110010001001000011
110111111111111111111011111111011111101111111100101010110010001001001001111011111
101111111111111111110010111111111111110101011110010000000

EMS 1% (8h) 1

000000011011000001110000000000000000100000000000000000000000000000000000000000
110110000011100000000000000000100000000000000000000000000000000000000000001101111
101111111111111111111010110000011100000000000110000000000

CONTROL

00000111101111111111111111111111111101111111111111001010101100100000010010011
11011111111111011111111111111010000000000000000000000000000000000000111101100
00000001111111111111010111111111101100000111100000000100